The role of bone marrow-derived mesenchymal stem cells in lymphoma tumor growth

Roorda, Berber Dorienke

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Berber D. Roorda

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Stellingen behorend bij het proefschrift

The role of bone marrow-derived mesenchymal stem cells in lymphoma tumor growth

1 Bone marrow-derived mesenchymal stem cells can promote tumor growth by direct interactions with tumor cells as well as by modulating the tumor microenvironment. *This thesis*

2 VEGF-A promotes lymphoma tumor growth by activation of STAT proteins and inhibition of $p27^{KIP1}$ via paracrine mechanisms. *This thesis*

3 The use of MSCs in clinical practise can be both a risk and benefit since MSCs may be able to home to tumors and contribute to tumor growth, while they are also being investigated as therapeutic vehicles to inhibit tumor growth. *This thesis*

4 Although bone marrow-derived MSCs seem to lack specific tropism to lymphoma tumors, their contribution to lymphoma growth may not be negligible. *This thesis*

5 "The line between profound talent and profound disability seems to be really a surprisingly thin one. " *Daniel Tammet, an autistic savant*

6 "Judo is niet zozeer een gevechtskunst, als wel het basisprincipe van menselijk gedrag." *Jigoro Kano, de grondlegger van het Judo*

7 Sinds de opkomst van de 'opzoek'-cultuur in het onderwijs is onthouden een vergeten kunst geworden

8 In tegenstelling tot de kindertijd en de puberteit is criminaliteit niet aan leeftijd gebonden

9 Het leggen van eieren is niet enkel weggelegd voor vogels maar ook voor onderzoekers

10 Kleding die niet gepast is, is niet per definitie ongepast

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Voor oma Grietje Bruinsma (1927-2008)

Omdat ik de kans wel heb gekregen

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Chapter 1

General introduction and aim of the thesis

General introduction

Non-Hodgkin lymphoma (NHL) comprises a diverse group of hematological disorders originating in the lymphatic system (fig 1A). NHL is a clonal disorder of the immune system that results from the transformation of immature lymphoid progenitor cells at a particular stage of development to malignant lymphoid progenitor cells. Malignant lymphoma creates tumors that enlarge the lymph nodes and/or grow in other sites that are part of the immune system, for example the skin, spleen, or bone marrow.

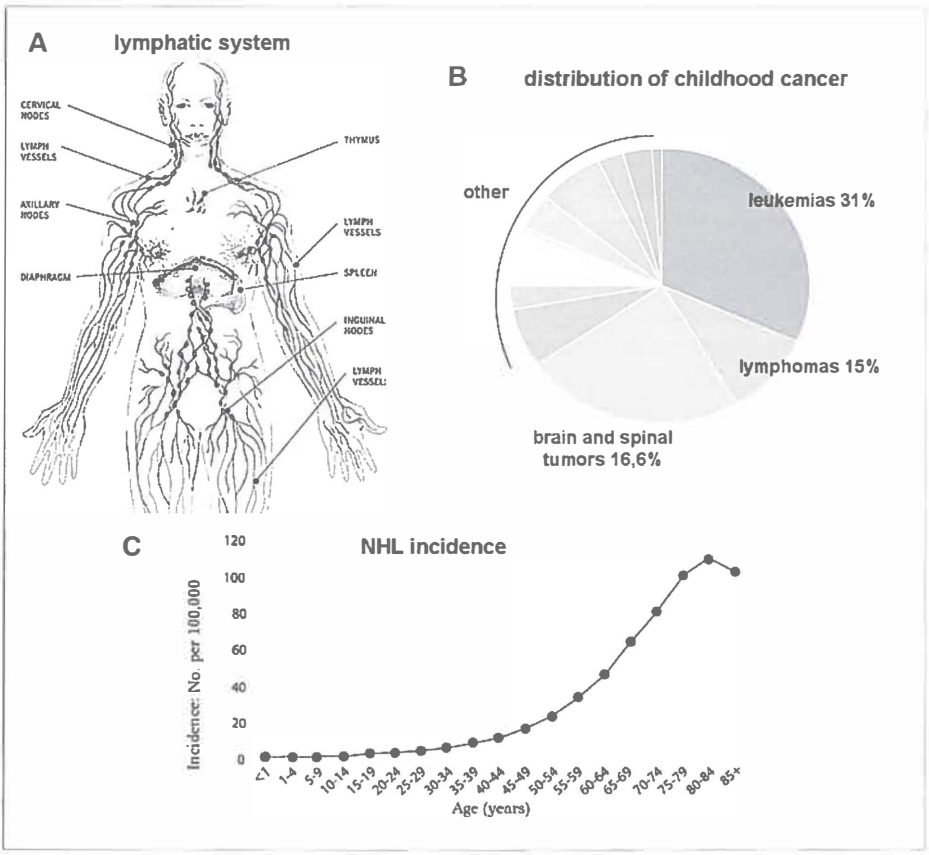

Figure 1: Non-Hodgkin lymphoma (NHL). *A) The lymphatic system; B) Percentage distribution of chilhood cancer; C) Incidence of NHL. The horizontal axis shows 5-year age intervals. The vertical axis shows the frequency of new cases of non-Hodgkin lymphoma each year per 100,000 people, by age-group.*

Lymphoma (about 15%; NHL and Hodgkin lymphoma) is the third most common cancer in children, following leukemia (31%) and brain and spinal tumors (16,6%) (1) (fig 1B). The NHL of children are a heterogeneous group of tumors, with Burkitt's and Burkitt-like tumors predominating among 5-14 year olds, and with diffuse large cell lymphomas being the most common subtype among 15-19 year olds (1;2). The incidence of NHL increases with age; about 2,2 cases per 100.000 persons occur in individuals under the age of 20, while from age 80 to 84 the rate increases more than 54-fold to 118,1 cases per 100.000 persons (1) (fig 1C). In The Netherlands, the number of newly diagnosed NHL patients is expected to increase with 36,1% between 2000 and 2020 due to the demographic development of the Dutch population (3). Despite improvement of treatment strategies in the last decades, about 20,3% of children and about 31% of adults with NHL still die from their diseases within 5 years after diagnose (1), underlining the relevance of more research on NHL growth and progression.

It is becoming increasingly apparent that the tumor microenvironment plays a determining role in cancer development, including lymphoma. Important components of the tumor microenvironment are stromal cells, such as endothelial cells, pericytes, smooth muscle cells, macrophages, mast cells, adipocytes and fibroblasts. In human, Ruan et al. reported differential stromal compositions in aggressive and indolent subtypes of Non-Hodgkin lymphoma (4), providing a novel perspective into the complex interplay between different types of stromal cells within the lymphoma microenvironment. Tumor stromal cells are not passive bystanders in tumor growth but are thought to actively participate in the promotion of tumor development and progression by secreting a variety of growth factors, chemokines and enzymes. Factors produced and secreted by tumor stromal cells contribute to remodelling processes in the tumor microenvironment to provide optimal support to tumor progression.

Experimental and clinical studies have revealed that tumor stromal cells can be bone marrow-derived. For example, bone marrow-derived endothelial progenitor cells (EPCs) were found to be recruited in response to proangiogenic signals and contribute to tumor angiogenesis in a murine xenograft model of aggressive B-cell

lymphoma (5). Furthermore, in a pilot study of six human patients with spontaneous cancers following bone marrow transplantation from opposite sex donors, an average of 4.9% bone marrow contribution to the tumor vasculature was noted, with Hodgkin's lymphoma exhibiting the greatest contribution of 12.1% (6). Moreover, it has been postulated that non-hematopoietic mesenchymal stem cells (MSCs) are the bone marrow-derived progenitors of tumor stromal pericytes, fibroblasts and smooth muscle cells (7-9). Fibroblasts and pericytes both have been shown to play important roles in tumor growth and progression (10;11), indicating a potential crucial role for bone marrow-derived MSCs in the development of cancer. Indeed, at the start of our research project, the first papers were published showing that bone marrow-derived MSCs have the potential to facilitate tumor growth (in murine adenocarcinoma and melanoma models (12), and a human colon cancer model (13)). However, the mechanism by which MSCs would promote tumor growth remained unclear.

Identification of critical components of the tumor microenvironment and critical interactions of tumor cells with their microenvironment will lay the foundation for research on novel and additional strategies in the treatment of aggressive lymphoma. Understanding how the tumor microenvironment would influence tumor growth could open the way toward therapeutic strategies targeting the molecular mechanisms that underlie relevant interactions between stromal cells and tumor cells. Successful targeting of such mechanisms could result in loss of stromal support and/ or functional isolation of tumor cells leading either to their eradication or their sensitization to conventional and new therapies.

Aim of the thesis

The aim of this thesis was to determine promotion of lymphoma tumor growth by bone marrow-derived mesenchymal stem cells (MSCs), to explore the mechanism by which MSCs would promote tumor growth, and to explore ways to inhibit the effects of MSCs on tumor growth.

In **chapter 2** an overview of the literature is presented regarding the currently known bone marrow-derived cell types that have been found to contribute to tumor growth with a special focus on bone marrow-derived MSCs. Potential underlying mechanisms of promotion of tumor growth by MSCs are discussed, as well as the potential of inhibiting bone marrow-derived cells and their utilization as cellular vehicles for selective delivery of cancer therapeutics as additional strategies in the treatment of cancer.

In **chapter 3**, we explored ways to inhibit the tumor growth-promoting effects of MSCs *in vitro* by testing the small molecule tyrosine kinase inhibitor PTK787/ZK 222584 (Vatalanib, PTK). We investigated the effect of PTK787/ZK 222584 on proliferation, survival, tube formation and migration of MSCs and on the outgrowth of MSCs from bone marrow mononuclear cells. In this study, we used Pepchip arrays as a novel tool to get a closer look to the effect of PTK787/ZK 222584 on the kinase activity profile of MSCs.

In **chapter 4**, we investigated the effect of MSCs on lymphoma tumor growth *in vivo*, using an earlier used mouse model (14) by determining tumor incidence, tumor weight and tumor vessel density. To gain more insight in the mechanism by which MSCs may promote tumor cell growth, *in vitro* co-culturing experiments were performed. In these experiments, we discriminated between potential effects induced by direct cell-cell contact interactions and/ or by the production of soluble factors. In addition, we investigated the effect of PTK787/ZK 222584 on MSC-promoted tumor growth in this *in vitro* co-culturing system as well as in our *in vivo* tumor model.

Bone marrow-derived MSCs are thought to be mobilized into the peripheral blood and to home to tumors in response to factors produced by the tumor, such as vascular endothelial growth factor A (VEGFA). In **chapter 5**, we assessed whether VEGFA overexpression by the tumor functions as a chemoattractant for circulating human BM-derived MSCs *in vivo*. We investigated direct homing of GFP-labelled MSCs to subcutaneous (s.c.) VEGFA overexpressing tumors and control tumors. We injected MSCs intravenously to mimic the physiological route of bone marrow-derived cells that may migrate towards tumor sites.

Both tumor cells and MSCs have been shown to produce VEGFA, which is the most important pro-angiogenic factor involved in normal and pathologic angiogenesis. In **chapter 6**, we investigated the interaction between tumor stromal cells and Daudi Burkitt lymphoma tumor cells in response to tumor-derived VEGFA. In this study, we used proteome profiler arrays as a novel tool to get more insight into the mechanism of VEGFA-promoted tumor growth as well as potential drugable targets for the treatment of lymphoma.

In **chapter 7**, the results of the different studies are summarized. The implications of this research are discussed in the context of current knowledge, and future perspectives arising from this research are presented.

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Chapter 2

Bone marrow-derived cells and tumor growth: Contribution of bone marrow-derived cells to tumor micro-environments with special focus on mesenchymal stem cells

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Abstract

Research has provided evidence that tumor growth depends on the interaction of tumor cells with stromal cells, as already suggested in 1889 by Paget. Experimental and clinical studies have revealed that tumor stromal cells can be derived from bone marrow (BM)-derived progenitor cells, such as mesenchymal stem cells (MSCs), which can be mobilized into the circulation and incorporate into tumor micro-environments. Many observations indicate that, in the tumor micro-environment, MSCs have several tumor growth promoting functions, including expression of growth factors, promotion of tumor vessel formation and creation of tumor stem cell niches. This review will discuss the currently known tumor growth promoting BM-derived cells and focus on the role of MSCs in modulating tumor micro-environments. In addition, we will discuss the potential of inhibiting BM-derived cells and their utilization as cellular vehicles for selective delivery of cancer therapeutics as additional strategies in the treatment of cancer.

Bone marrow-derived tumor stromal cells

In 1889, Stephen Paget proposed the concept that the micro-environment of a developing tumor is a crucial regulator of tumor growth in his famous 'seed and soil' hypothesis (1). In the next century, in 1971, Folkman *et al*. proposed the hypothesis that all tumor growth is dependent on the formation of new blood vessels (2). The ability of a tumor to progress from a non-angiogenic to an angiogenic phenotype was found to be crucial for the progression of cancer and was called the 'angiogenic switch' (3). In the last decades, it has become clear that tumor growth not only results from the interaction of tumor cells with stromal endothelial cells, but also with stromal fibroblasts, pericytes and inflammatory cells, as already suggested in 1889 by Paget. In mouse models as well as in humans, it was observed that tumor stromal cells could be derived from progenitor cells residing in the bone marrow (4-8). These bone marrow (BM)-derived progenitor cells can be mobilized into the circulation (5;9-13), migrate towards tumors, incorporate into the tumor micro-environment, and contribute to the growth of various tumors (4-8;14-18).

Bone marrow (BM)-derived tumor stromal cells including endothelial cells, fibroblasts and pericytes, are believed to originate from hematopoietic stem cells as well as non-hematopoietic stem cells residing in the bone marrow *(Figure 1)*. BM-derived tumor associated endothelial cells are derived from endothelial progenitor cells (EPCs) or EPC-like cells. True EPCs are believed to originate from hemangioblasts (19;20), common precursors for hematopoietic cells and endothelial cells. Alternatively, EPC-like cells are trans-differentiated monocytes or macrophages (21), non-hematopoietic multipotent progenitor cells (MAPCs) (22) or mesenchymal stem cells (MSCs) (23;24) expressing endothelial specific markers. The origin of BM-derived tumor associated fibroblasts and pericytes is not precisely known. It has been suggested that BM-derived fibroblasts and pericytes may originate from pericyte progenitors (25), and/ or from circulating fibrocytes that comprise 0.1-0.5% of the human mononuclear cell fraction in peripheral blood (26;27), and/ or from mesenchymal stem cells (28). Similarly, BM-derived progenitors of pericytes are suggested to originate from hematopoietic stem cells (25) and/ or non-hematopoietic mesenchymal stem cells (29).

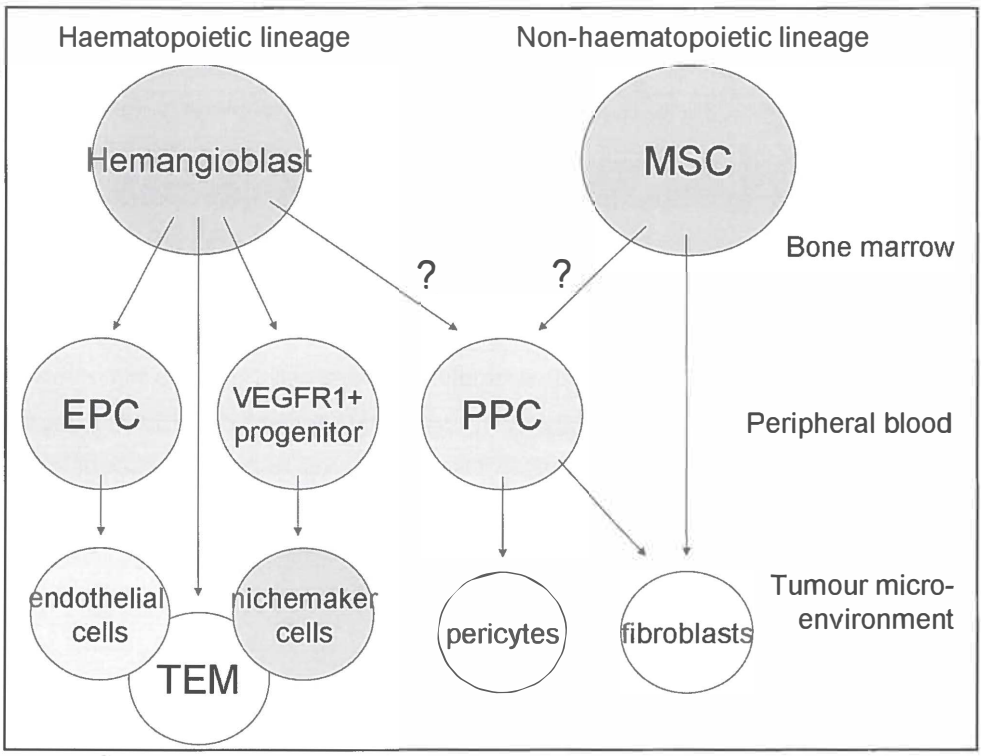

Figure 1: bone marrow-derived cells modulating the tumor micro-environment. *Bone marrow-derived tumor stromal cells including endothelial cells, TEMs, nichemaker cells, pericytes and fibroblasts are believed to originate from hematopoietic or non-hematopoietic stem cells residing in the bone marrow. Hematopoietic and non-hematopoietic stem cells provide progenitor cells, which can enter the peripheral blood and migrate towards tumor sites. EPCs, VEGFR1$^+$ progenitor cells and TEMs are thought to originate from hemangioblasts, common precursors for hematopoietic and endothelial cells. Endothelial cells are derived from EPCs after differentiation along the endothelial lineage, whereas TEMs and nichemaker cells are derived from hematopoietic progenitor cells after differentiation along the myeloid lineage. The origin of PPCs and their derivatives is less clear. PPCs may be derived from hematopoietic stem cells or non-hematopoietic MSCs. Pericytes and fibroblast may be derived from PPCs or directly from MSCs. (TEMs: Tie2-expressing monocytes, EPCs: endothelial progenitor cells, PPCs: pericyte progenitor cells, MSCs: mesenchymal stem cells).*

Endothelial Progenitor Cells

In 1997, Asahara *et al.* demonstrated that human peripheral blood contained cells able to differentiate into endothelial cells (13). He was the first to isolate endothelial progenitor cells (EPCs) from human peripheral blood using anti-CD34 monoclonal

antibodies. CD34-antigen, however, could not distinguish between immature EPCs and mature circulating endothelial cells (CECs), due to the fact that CD34 is expressed on both immature and mature endothelial cells. With the discovery of CD133, an antigen specifically identifying more immature EPCs, this distinction between EPC and mature endothelial cells could be made (30). EPCs are also characterized by expression of the receptor for vascular endothelial growth factor-2 (VEFGR2). Signalling through this receptor promotes EPCs to differentiate along the endothelial lineage and induces expression of endothelial-specific cell markers such as Von Willebrand factor (vWF) and vascular endothelial (VE)-cadherin (31).

The first proof-of-principle of EPC contribution to the tumor micro-environment was reported in 2001 by Lyden et al (5). In angiogenesis-defective Id-mutant mice transplanted with donor β-galactosidase-positive (β-gal$^+$) bone marrow from Rosa-26 mice, LacZ-staining (LacZ is the reporter gene that codes for β-galactosidase) revealed the presence of donor-derived bone marrow cells in tumor vessels. Approximately 90% of the tumor vessels expressing vWF were characterized as LacZ-positive demonstrating that BM-derived cells had incorporated into vessels associated with the tumors grown in these mice (5). Similarly, in humans previously transplanted with hematopoietic stem cells from a donor of the opposite sex, examination of secondary tumors revealed that 0.5–12% of tumor endothelial cells were donor-derived (6). However, the extent to which EPCs contributed to the generation of the tumor vessels has been highly variable in different studies; substantial (5), low (6;32;33), negligible defined as less than 0.1% (7;34-38), depending on the tumor type and grade (39). Thus, although the contribution of EPCs to tumor vasculature is obvious, the extent appears to be highly dependent on the experimental model.

Pericyte Progenitor Cells

In the tumor micro-environment, pericytes and endothelial cells are two interacting cell types forming blood vessels. Endothelial cells form the inner lining of the vessel wall, while perivascular cells (referred to as pericytes), vascular smooth muscle cells (vSMCs) or mural cells wrap around the vascular tube. Pericytes function through paracrine contact and cell-cell contact with endothelial cells and are believed to play

a role in the stabilization of blood vessels (40-42). One of the first studies proposing the existence of BM-derived tumor stromal pericytes came from Rajantie *et al.*, who observed high numbers of perivascular cells in tumor blood vessels in mice transplanted with green fluorescent protein-positive (GFP^+) bone marrow (7). Most of these perivascular cells were in close contact with the underlying endothelial cells and many of these cells expressed NG2 proteoglycan (NG2), a marker for developing pericytes (43). Subsequently, results from Song *et al.* suggest that these BM-derived tumor associated pericytes originate from BM-derived pericyte progenitor cells (PPCs) which in turn are derived from hematopoietic stem/ progenitor cells (25). In RIPTag2 mice (a model for pancreatic tumorigenesis) transplanted with GFP^+ bone marrow, Song *et al.* demonstrated that GFP^+ PPCs are characterized by the expression of platelet-derived growth factor receptor-beta (PDGFRβ) and that these PPCs are able to differentiate into mature pericytes expressing the markers NG2, desmin and alpha-smooth muscle actin (α-SMA) (25). In addition, about 23% of $PDGFR\beta^+$ PPCs were found to be immunoreactive for stem cell antigen-1 (Sca1), which is expressed on murine hematopoietic stem cells. In $PDGFR\beta^+$ PPCs modest mRNA expression of the stem/progenitor cell marker c-Kit was detected by RT-PCR analysis. These data suggest that $PDGFR\beta^+$ PPCs can be derived from BM-derived hematopoietic stem/ progenitor cells that are recruited to the angiogenic vasculature of pancreatic tumors and are able to differentiate into mature pericytes.

$VEGFR1^+$ Progenitor Cells

In addition to BM-derived PPCs, BM-derived myeloid cells, characterized by expression of the receptor for vascular endothelial growth factor-1 (VEGFR1), were also found to incorporate into the tumor micro-environment by localizing to perivascular sites. Lyden *et al.* transplanted vascular endothelial growth factor (VEGF)-mobilized cells collected from the peripheral circulation of $\beta\text{-gal}^+$ Rosa-26 mice into lethally irradiated angiogenesis-defective Id-mutant mice. Transplantation of VEGF-mobilized cells resulted in restoration of angiogenesis and tumor growth of the implanted B6RV2 leukemia tumor cells in Id-mutant mice. Analysis of tumors demonstrated the presence of vWF^+LacZ^+ vessels, decorated with $VEGFR1^+LacZ^+$ mononuclear cells. Furthermore, in tumors of mice treated with antibodies against VEGFR1, diminished vascular investment with perivascular cells was observed (5).

Because of the common perivascular localization of VEGFR1$^+$ cells and BM-derived PPCs, it has been suggested that VEGFR1$^+$ cells differentiate into pericyte-like cells and play a role similar to pericytes in the tumor micro-environment (5). Incorporated VEGFR1$^+$ cells are not known to express the pericyte markers NG2 and α-SMA. However, VEGFR1$^+$ cells co-stained for myeloid-specific markers including monocyte macrophage (MOMA) and CD11b (5), suggesting a myelomonocytic origin. This finding indicates that the pericyte-like cells derived from VEGFR1+ cells are nevertheless different from the pericytes derived from PPCs, which do not express myeloid-specific markers (25).

Interestingly, Kaplan *et al.* reported that VEGFR1$^+$ BM-derived cells have a functional role in directing metastasis (44). In mice, Kaplan *et al.* analysed the fate of β-gal$^+$ or GFP$^+$ BM-derived cells following intradermal injection of lewis lung carcinoma (LLC) or B16 melanoma tumor cells, which are known to metastasize to the lungs. Fourteen days after tumor implantation, but already before the arrival of tumor cells, cluster formation of β-gal$^+$ BM-derived cells was detected in the lungs. After 18 days, individual DsRed-tagged tumor cells, associated with pre-existing BM-derived cell clusters, were visible and progressed to micrometastases by day 23. Clusters induced by either tumor type expressed VEGFR1 and further characterization revealed that subsets of VEGFR1$^+$ BM-derived cells co-expressed the stem/progenitor cell antigens CD133, CD34 and c-Kit, suggesting that these cells may comprise VEGFR1$^+$ hematopoietic progenitor cells. Treating animals with VEGFR1 neutralizing antibodies entirely eliminated the initiating clusters and prevented metastasis. Because of the observation that BM-derived VEGFR1$^+$ cells have the capability to initiate and maintain pre-metastatic niches, they have been called nichemaker cells (44). Moreover, in human tissues from patients with malignancies VEGFR1$^+$ clusters were observed in both primary tumors and metastatic tissue and increased numbers of VEGFR1$^+$ clusters were observed in common sites of metastasis before tumor spread, suggesting the potential of this tissue as a future site for metastasis (44). All together, these data indicate a clinical relevance to inhibit BM-derived VEGFR1$^+$ cells to prevent metastasis in human patients with malignancies.

Tie2-Expressing Monocytes

Tumor progression can be influenced by tumor-associated macrophages (TAMs) and $CD11b^+$ myeloid cells, which originate from hematopoietic progenitor cells differentiated along the myeloid lineage. TAMs and $CD11b^+$ myeloid cells are overlapping and functionally heterogeneous cell populations. In 2005, De Palma *et al.* demonstrated that a specific subset of these cells, called Tie2-expressing monocytes (TEMs), accounts for most of the pro-angiogenic activity of myeloid cells in tumors (4). It was reported that TEMs recruit to spontaneously arising mouse pancreatic tumors and orthotopic xenografts of human glioblastomas (4). In RIPTag2 mice transplanted with bone marrow cells expressing GFP under the control of the Tie2-promotor, Tie2-GFP^+CD11b^+ TEMs were selectively present in the pancreatic tumors, and not in the surrounding tissue. Many TEMs had a perivascular location similar to that of pericytes; however, TEMs could be distinguished from pericytes since they did not express NG2 and α-SMA. Staining of tumors showed that the wide majority of TEMs expressed basic fibroblast growth factor (bFGF), a potent pro-angiogenic molecule, suggesting that TEMs promote angiogenesis via release of bFGF. In another experiment, De Palma *et al*. studied tumor growth after elimination of TEMs, using nude mice transplanted with BM-derived lineage-negative cells expressing the thymidine kinase (TK) suicide gene. Treatment with ganciclovir (GCV) in order to eliminate TEMs (the enzyme derived from the TK gene converts GCV to a form that is toxic for the cell), starting one day after implantation of human glioma cells in the striatum, resulted in almost avascular tumors, whereas the tumors of untreated mice were highly angiogenic. This result indicates that elimination of TEMs from the tumor micro-environment severely impairs tumor vessel formation at least in orthotopic gliomas (4).

Recently, Venneri *et al*. identified Tie2-expressing monocytes in human peripheral blood (45). These TEMs accounted for 2-7% of blood mononuclear cells of healthy donors and were also observed in the blood of cancer patients (1.8% to 10.1% of mononuclear cells). Further studies are required to assess whether TEM frequency in cancer patients correlates with clinical parameters. Intriguingly, in cancer patients, TEMs represented the main monocyte population in tumors, whereas they were hardly detected in non-neoplastic tissues (45). To investigate whether human TEMs

have pro-angiogenic activity, Venneri *et al.* co-injected TEMs isolated from human peripheral blood with U87 human glioma cells in nude mice and analysed tumor vascularization at a very early stage of tumor growth (5-7 days after injection). In tumors derived from the injection of U87 cells alone, $CD31^+$ blood vessels were exceedingly scarce, indicating that angiogenesis had not yet started at this early time of tumor growth. In contrast, tumors co-injected with TEMs were larger and much more vascularized. These results indicate that, among human blood monocytes, the TEM subset is specifically endowed with the ability to enhance angiogenesis.

Mesenchymal Stem Cells

Recently, mesenchymal stem cells (MSCs) have been added to the list of BM-derived cells that contribute to the tumor micro-environment thereby influencing tumor growth and progression (figure 2). BM-derived MSCs are non-hematopoietic stem cells characterized by the expression of a large number of adhesion molecules and stromal cell markers (CD73, CD105, CD44, CD29, CD90) in the absence of hematopoietic markers (CD34, CD45, CD14) and endothelial markers (CD34, CD31, vWF) (46-49). MSCs express various cytokine and growth factor receptors and produce a large number of cytokines, growth factors and extracellular matrix proteins (fibronectin, vimentin, laminin). MSCs have the potential to differentiate along osteogenic, adipogenic and chondrogenic lineages when placed in the appropriate environments (46-49) and this differentiation plasticity makes MSCs the ultimate candidates for future utilization in cell therapy and tissue regeneration. Opportunities and challenges for clinical use of MSCs in cardiac repair are reviewed by Atsma *et al.* (50). Other therapeutic applications of MSCs are discussed by Brooke *et al.* (51). In the adult bone marrow, MSCs are progenitors of bone marrow stromal fibroblasts, which play a crucial role in supporting hematopoiesis by providing hematopoietic progenitors necessary cytokines and cell-cell contact mediated signals to self renew or differentiate (47;52).

There is now good evidence that BM-derived cells contribute to fibroblast populations in tumor stroma (8;28). Direkze *et al.* showed that approximately 25% of α-SMA-positive myofibroblasts and also some vimentin-expressing fibroblasts were found to be derived from the bone marrow in pancreatic tumors (28). Experimental evidence

of fibroblast recruitment to tumor stroma in mice comes from the demonstration that intraperitoneously injected as well as intravenously injected human BM-derived β-gal-transduced MSCs preferentially localize within tumor stroma but not within the normal tissue stroma (53;54). MSC-derived stromal fibroblasts have been shown to favour tumor growth and progression of various xenografts in mice. Ramasamy *et al.* observed that 75% of mice injected with a mixture of tumor cells and cultured MSCs developed a tumor, whereas 12% of animals receiving tumor cells alone showed signs of tumor growth (17). In addition, earlier onset of tumor development (16;55) and increased tumor incidence (15;18) was observed in mice injected with a mixture of tumor cells and MSCs as compared to tumor cells alone.

It is not precisely known how MSCs influence tumor growth. Djouad et al. proposed that the immunosuppressive properties of MSCs allow proliferation of tumor cells otherwise rejected by immunocompetent recipients (15). Immunomodulatory properties of MSCs are excellently reviewed in Blood by Nauta *et al.* (56). In addition, research from the last decade indicates that MSCs may actively promote tumor growth by modulating the tumor micro-environment, for example by stimulating tumor vessel formation (18;23).

Tumor micro-environmental modulation by MSCs

BM-derived MSCs are co-recruited along with BM-derived hematopoietic and endothelial progenitor cells to establish a supportive tumor stroma. Tumor stromal cells including pericytes, endothelial cells, fibroblasts, macrophages and other immune cells have been shown to contribute to the malignant progression of tumors. MSCs are thought to modulate the tumor micro-environment by different mechanisms. For example, MSCs may be involved in the process of new vessel formation. In mice, systemically administered MSCs engrafted to wounds and in these wounds levels of pro-angiogenic factors were increased. MSC-treated wounds showed accelerated wound closure and increased vessel formation (57). Tumor formation is often accompanied by a response that closely resembles wound healing. As in responses to injury, tumorigenesis also entails production of pro-angiogenic growth factors and formation of new blood vessels that render a tumor as a 'wound that never heals' (58). Interestingly, we found increased densities of immature

(desmine-negative) Factor VIII-positive vessels in tumors inoculated with MSCs as compared with tumors inoculated without MSCs indicating a role for MSCs in promoting tumor vessel formation (unpublished data). Alternatively, it has been hypothesized that MSCs may form cancer stem cell niches supporting the survival of tumor stem cells and protecting them from drug-induced apoptosis (17). Both proposed roles of MSCs in modulating the tumor micro-environment will be discussed below.

Role in tumor vessel formation

The mechanism by which MSCs would contribute to tumor vessel formation is at least complex and not precisely known. First, MSC-derived fibroblasts are thought to promote tumor growth directly by the production of growth factors acting on tumor cells, or indirectly by the production of more specific pro-angiogenic growth factors *(Figure 2)*, such as VEGF, platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and stromal-derived factor-1 (SDF1) (59). Endothelial cells express receptors for and can be activated in response to these MSC-derived angiogenic factors. It has been demonstrated that media collected from MSC cultures promoted in vitro proliferation, migration and survival of endothelial cells (59;60) as well as in vitro tube formation (61). Thus, MSCs are very likely to be involved in the creation of a pro-angiogenic tumor micro-environment.

Secondly, MSC-derived fibroblasts are thought to have the capacity to (trans)-differentiate into endothelial-like cells and pericyte-like cells, which would stimulate tumor growth by the formation and stabilization of tumor blood vessels *(Figure 2)*. MSCs indeed have the capability to aquire endothelial-like characteristics in pro-angiogenic environments (23;24;62;63). Human BM-derived MSCs, negative for endothelial specific markers, when cultured in medium supplemented with fetal calf serum (FCS) and VEGF, differentiated towards endothelial-like cells after seven days (24). The MSCs acquired several distinct characteristics of mature endothelial cells, such as VEGFR1, VEGFR2, VE-cadherin, vascular cell adhesion molecule-1 (VCAM1) (24) and vWF (62) expression. Furthermore, we (unpublished data) and others showed that MSCs have an inherent capacity to rapidly form in vitro tube-like structures when cultured on 3D-matrigels (64). Endothelial differentiation of MSCs is

thought to be regulated by VEGF-induced PDGF-receptor signaling since MSCs lack expression of VEGF-receptors (65). The role of PDGF-receptors in regulating the vascular cell fate of MSCs has been reviewed by Ball *et al.* (66).

Moreover, research has indicated that MSCs may physically incorporate in to the vasculature *in vivo*. In mice, it was reported that MSCs formed tube-like structures in implanted matrigel plugs (63). It has been observed that MSCs incorporated into newly formed vessels after myocard infarction and displayed endothelial phenotypes in pigs (67) and dogs (68). Furthermore, in mice xenograft models, it was observed that MSCs co-injected with U-87 glioblastoma tumor cells incorporated into tumor neo-vasculature and were able to differentiate into CD31-positive endothelial-like cells (64). In addition, systemically injected MSCs were shown to localize and engraft into tumors, where they proliferated, differentiated into vWF- and CD31-positive endothelial-like cells and formed a significant fraction of the tumor micro-environment (69). Besides differentiating into endothelial cells, it has been suggested that MSCs contribute to the vasculature by (trans)-differentiating into pericyte-like cells. Several studies have demonstrated that MSCs express α-SMA and secrete extracellular matrix (ECM) proteins, which are inherent characteristics for smooth muscle cells (70;71), equivalents of pericytes. It was observed that MSCs incorporated into newly formed vessels after myocard infarction displayed vascular smooth muscle cell phenotypes in pigs (67) as well as in dogs (68). Overall, these data indicate a role for MSCs in modulating the tumour micro-environment by its production of several growth factors as well as by a (trans)-differentiation in endothelial-like and pericyte-like cells.

Creation of cancer stem cell niches

MSC-derived stromal fibroblasts are components of hematopoietic stem cell niches and consequently play a crucial role in supporting normal hematopoiesis (72-74). They provide hematopoietic progenitors the necessary cytokines and cell contact mediated signals to self renew or differentiate (47;52). Similarly, it has been proposed that MSCs may also form cancer stem cell niches by which the survival of tumor cells is supported (17). Ramasamy *et al.* showed that human MSCs exhibit a potent anti-proliferative activity in vitro on different tumor cell lines; MSCs induced

downregulation of cyclin D2 in tumor cells associated with tumor cell cycle arrest in the G_1 phase and a reduction of tumor cell apoptosis, indicating that the tumor cells may enter a more quiescent state (17). However, this effect was transient and when assessed *in vivo*, it resulted in facilitation of tumor engraftment and growth. In fact, mice injected with tumor cells in the presence of MSCs showed a much faster tumor growth than those which did not receive any MSCs (17). The quiescent state of stem cells is of critical importance in preserving their self-renewal abilities and promoting their survival, because when stem cells are highly proliferative their longevity is dramatically reduced (75). It is tempting to speculate that the presence of MSCs in tumor micro-environments may promote the survival of cancer stem cells by protecting them from apoptosis, resulting in increased tumor incidences and enhanced tumor growth *in vivo.* Pilot data, confirming this hypothesis have been published very recently in Leukemia; Ning *et al.* found a correlation between co-transplantion of MSCs along with allogenic hematopoietic stem cells and a higher recurrence rate in hematologic malignancy patients (76). Furthermore, there is some evidence that the interaction of tumor cells with bone marrow stromal cells may even protect tumor cells from drug-induced apoptosis (17;77). On adhesion to fibronectin on bone marrow stromal cells, mediated by adhesion molecules of the very late antigen-4 (VLA4) integrin type on tumor cells, tumor cell lines and acute myeloid leukaemia (AML) cells from human patients become resistant to spontaneous or drug-induced apoptosis (77). These data are consistent with the finding that, in a mouse model of AML, the combination of anti-VLA4 antibodies with cytarabine (AraC) induced long-term remissions, whereas AraC alone caused only a minor prolongation of survival (77).

Presence of mesenchymal/fibroblast-like cells in peripheral blood

A possible contribution of BM-derived MSCs to distant solid tumors implies that they should be demonstrable in peripheral blood under certain circumstances. In mice, Kawada *et al.* showed that MSCs indeed can be mobilized into the peripheral blood by granulocyte colony stimulating factor (G-CSF) and that these cells can migrate and incorporate into the myocardium and differentiate into cardiomyocytes after infarction (11). Moreover, in rats, Bittira *et al.* observed that MSCs migrate spontaneously from the bone marrow toward the infarcted myocardium (78). In

humans, MSCs have been detected in peripheral blood from breast cancer patients after growth factor mobilization (79;80), and in non-mobilized blood from healthy subjects (81;82). Other groups were, however, not able to detect circulating MSCs (83;84). These discrepancies may be attributed to differences in mobilization procedures, different growth factor regimens, leukapheresis procedures, cell preparation or culture media. Taken together, these data support the existence of a small population of circulating MSCs in the peripheral blood of humans, the isolation of which is clearly difficult and subject to variation.

Mobilization of bone marrow-derived progenitor cells

In general, BM-derived cells can be mobilized by factors produced and excreted by tumors. Tumor-derived factors, including VEGF, angiopoietin-1 (Ang1), SDF1, and granulocyte macrophage colony stimulating factor (GM-CSF) have been implicated in the mobilization of EPCs, $VEGFR1^+$ cells as well as MSCs out of the bone marrow into circulation (5;9-13). Circulating BM-derived cells are thought to migrate towards tumors along gradients of chemokines produced and excreted by tumors (85). For example, EPCs have been shown to migrate along gradients of VEGF (9;13) and SDF1 (86;87) produced by tumors, and TEMs have been shown to migrate along a gradient of angiopoietin-2 (Ang2), a Tie2-ligand that is released by activated endothelial cells in tumors (45). Similarly, PDGFβ, a PDGFRβ-ligand that is released by activated endothelial cells, may function to recruit pericyte progenitors to tumors (40;88). Mechanisms of homing and integration of progenitor cells into the tumor micro-environment are largely unknown. Similar to the homing and extravasation of leukocytes, homing and transmigration of BM-derived cells is thought to be regulated by adhesion molecules and integrines (89). For example, it has been shown that beta2-integrin facilitates homing and trans-endothelial migration of EPCs to tumor sites (90) and *in vivo* blocking experiments have provided evidence that initial arrest of BM-derived cells in the tumor vascular bed is mediated by E-selectin and P-selectin (91). Once incorporated in the tumor micro-environment, BM-derived cells themselves are thought to release growth factors enhancing the recruitment of even more BM-derived cells in a self-amplifying loop (92).

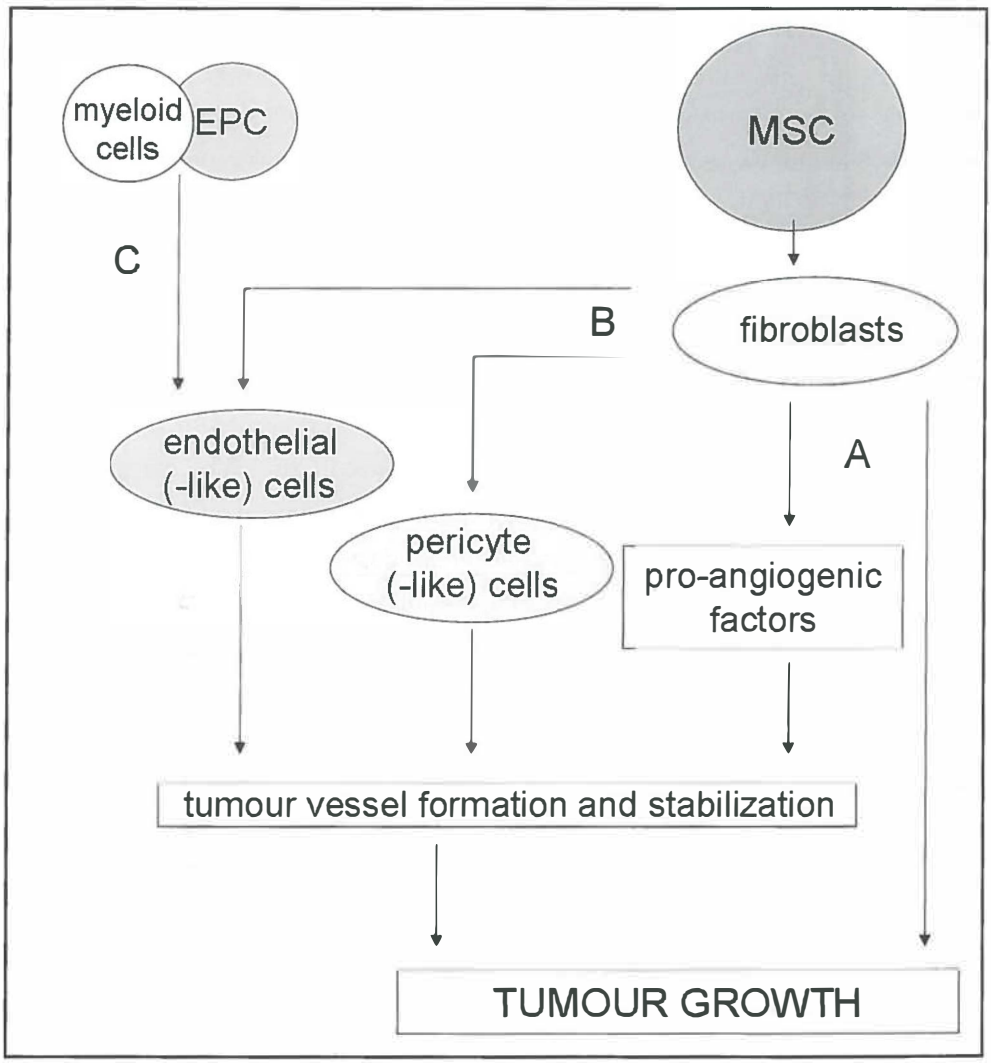

Figure 2: the role of mesenchymal stem cells in modulating the tumor micro-environment. *MSCs are precursors for tumor stromal fibroblasts. A) MSC-derived fibroblasts are thought to promote tumor growth directly by the production of growth factors acting on tumor cells, or indirectly by stimulating tumor vessel formation by the production of pro-angiogenic growth factors. B) MSC-derived fibroblasts are thought to have the capability to (trans)-differentiate into endothelial-like cells and pericyte-like cells, which would stimulate tumor growth by the formation and stabilization of tumor blood vessels. C) Genuine endothelial cells originate from EPCs, whereas endothelial-like cells are derived from progenitor cells others than EPCs, including transdifferentiated monocytes/ macrophages or MSC-derived fibroblasts, which have acquired endothelial characteristics, such as tube formation abilities and endothelial marker expression. (MSCs: mesenchymal stem cells, EPCs: endothelial progenitor cells)*

Inhibition of bone marrow-derived tumor growth promoting cells

Research has shown that BM-derived cells have the capacity to functionally contribute to the growth of various tumors. As malignant progression of tumors is dependent on the interaction of tumor cells with their micro-environment, inhibition of tumor growth-promoting BM-derived cells may provide an efficient additional strategy to slow down tumor growth. Indeed, animal studies show that selective elimination of tumor growth-promoting BM-derived cells by suicide genes or neutralizing antibodies results in reduced tumor growth (4;5;25;93;94). Although results from animal studies are very promising, we are a long way off from being able to target tumor growth-promoting BM-derived cells in human patients with malignancies. It is not known whether inhibition of tumor growth-promoting BM-derived cells will be effective for all different types of tumors seen in human patients with malignancies. For example, little is known about which tumor types are most dependent on growth induced by BM-derived cells. It has been shown that the initial growth of lymphomas is fully dependent on incorporation of EPCs into the tumor vasculature, whereas the initial growth of Lewis lung carcinoma is only partially dependent on the recruitment of EPCs (95). Furthermore, it is not clear whether BM-derived cells are only required for (clinically undetectable) early stages of tumor growth or also for the maintenance of established tumor vessels at later stages of tumor growth. Finally, it will be very hard to target solely BM-derived cells, because they lack specific markers discriminating them from other cell-types. Further research is needed to investigate under what circumstances it would be recommended and feasible to inhibit tumor growth-promoting BM-derived cells.

Nevertheless, advantage could be taken from common functional markers expressed by both BM-derived cells and other tumor stromal cells to target the tumor micro-environment. Most research has focussed on targeting tumor vessel formation. For example, Lyden *et al.* investigated tumor growth in mice treated with neutralizing antibodies against VEGFR1, VEGFR2 or a combination of the two antibodies. Histological examination demonstrated that tumors of mice treated with antibodies against VEGFR2 showed decreased vessel densities, whereas tumors of mice treated with antibodies against VEGFR1 showed diminished vascular investment with perivascular cells consistent with mutually supporting roles for EPCs and $VEGFR1^{+}$

cells in new vessel formation and vessel stabilization respectively. Importantly, a combination of neutralizing antibodies against both VEGFR2 and VEGFR1 completely blocked tumor growth and induced tumor necrosis (5). Similarly, in RIPTag2 mice, tumor growth was reduced after treatment with an antibody against PGDFRβ, resulting in reduced numbers of tumor stromal pericytes by 70-89% as compared with untreated controls (25). Note that, in these studies designed to target BM-derived cells, other tumor stromal cells were also affected by the neutralizing antibodies used. Tyrosine kinase inhibitors, such as the VEGFR2-inhibitor PTK787/ZK 222584 and PDGFR-inhibitors SU6668 or STI571/Gleevec, are another class of drugs which can be used to target the tumor micro-environment and subsequent tumor growth. Bergers *et al.* showed that a combination of SU6668 or STI571/Gleevec with the VEGFR-inhibitor SU5416 was found to be most effective in reducing tumor growth, affecting both tumor pericytes and endothelial cells (96). Overall these data suggest that, in addition to conventional therapies directed towards tumor cells themselves, the combined targeting of tumor growth-promoting BM-derived cells and other components of the tumor micro-environment may improve the efficacy of cancer therapies.

Use of bone marrow derived cells for targeted therapy

As BM-derived cells are endowed with the capacity to home to the tumor micro-environment, they may be promising targets for the selective delivery of cancer therapeutics. For example, transplantation of genetically modified BM-derived cells may represent a vehicle for the transport of gene therapy to tumors in the future. Various types of BM-derived cells have been shown to preferentially home to tumor tissues, and when prepared with suicide genes, to reduce tumor growth (54;94;97). In 2003, Ferrari *et al.* genetically engineered ex vivo BM-derived EPCs to express the thymidine kinase (TK) suicide gene using retrovirus-mediated gene transfer (93). Genetically labelled EPCs were transplanted into sublethally irradiated tumor-bearing mice, and were found to migrate to, and incorporate within the angiogenic vasculature of growing tumors. Treatment with ganciclovir (GCV) resulted in significant tumor necrosis in animals that had previously been given TK-expressing EPCs, with no systemic toxicity (93). Furthermore, Wei and colleagues demonstrated that mouse embryonic EPCs stably expressing a suicide gene were able to reduce

tumor volume and the number of lung metastases, and prolong the lifetime of mice with multiple established lung metastases (94). Similarly, in mice previously transplanted with Tie2-TK BM-derived lineage-negative cells, De Palma *et al.* showed that treatment with GVC in order to eliminate TEMs resulted in smaller brain tumors than brain tumors in untreated mice, and tumor necrosis was dramatically increased in GCV-treated as compared to tumor necrosis in untreated mice (4). These results show that targeting of tumors using BM-derived EPCs or TEMs genetically engineered with suicide genes effectively reduces tumor growth in the described animal models, indicating that targeted therapy using BM-derived cells indeed would be a feasible approach for the treatment of certain cancers. This approach may overcome the extensive metabolism and toxicity associated with some biologic agents and could serve as a versatile tool for manipulating the extracellular milieu of malignant cells.

The use of EPCs or TEMs as cellular vehicles for the delivery of pharmaceuticals, however, is not so obvious. It is hampered by the fact that it depends on the isolation and purification of a large number of vehicle cells from the bone marrow or the circulation and optimal culturing conditions for clinical scale production of vehicle cells. Under steady state conditions, however, the number of EPCs and TEMs in the bone marrow or the circulation is very low. In addition, it is still difficult to isolate and purify EPCs and, although culturing conditions have been optimized for EPCs, these are still not optimal for large scale production. In contrast, MSCs can be very easily isolated from human bone marrow, and propagated and manipulated in vitro (98). Along with their selective migration properties towards tumor sites, MSCs would be the most promising cellular vehicles for the selective delivery of cancer therapeutics in the future. In rats, Nakamura *et al.* showed that intra-tumoral inoculation of MSC-IL2 (rat MSCs modified to produce human interleukin-2 (IL2)) prolonged mean survival time and reduced tumor volume compared to rats injected with MSC-GFP control cells (99;100). Similar results were obtained in a murine melanoma model by Elzaouk *et al.*, who showed that intra-tumoral application of MSCs stably transduced with a retroviral vector expressing interleukin-12 (IL12) resulted in a pronounced retardation of tumor growth and led to the prolonged survival of the mice compared to

mice injected with MSC control cells (101). These data show that genetically engineered MSCs can be used to produce factors that reduce tumor growth.

First evidence that MSCs can be used as systemically injected cellular vehicles for the selective delivery of cancer therapeutics came from Studeny *et al.;* in mice, it was demonstrated that intravenously injected MSC-IFNβ cells (MSCs modified to produce interferon-beta (IFNβ)) selectively migrate towards tumor micro-environments. Local production of IFNβ in tumors inhibited malignant cell growth and prolonged animal survival, whereas systemic levels of IFNβ supplied by the same number of subcutaneously injected MSC-IFNβ cells had no effect (54;102). Similarly, it has been shown that intravenously injected MSCs modified to produce natural killer transcript-4 (NK4; an antagonist of hepatocyte growth factor) (103) or fractalkine (CX3CL1;a chemokine important for the mobilization of NK-cells and T-cells) (104) inhibit the development of lung metastases in mice and prolong the survival of mice bearing lung metastasis. Furthermore, Kucerova *et al.* achieved significant inhibition of tumor growth by intravenously administered adipose tissue-derived MSCs expressing a pro-drug converting gene in mice treated with the drug 5-fluorouracil (5-FC) (105). Recently, Stoff-Khalili *et al.* used MSCs as a vehicle for targeted delivery of conditional replicating adenoviruses (CRAds) to lung metastases of breast carcinoma to study the utility of MSC for virotherapy, a novel therapeutic approach for the treatment of cancer in which replicating virus itself is the anticancer agent (106). Taken together, these data show that MSCs may be the most promising BM-derived cell type that can function as delivery vehicle for a cell-based targeted cancer gene therapy in the future.

Conclusions and future perspective

In conclusion, today, there is no doubt that BM-derived cells have the capacity to functionally contribute to tumor growth. BM-derived cells can be mobilized from the bone marrow into the peripheral blood, migrate towards sites of tumor formation and incorporate into the tumor micro-environment. Although it is highly variable in different animal models, the contribution of BM-derived cells to the tumor micro-environment seems to be essential. Further research is needed to determine the extent to which bone marrow derived cells contribute to the growth of different types

of tumors and whether it is feasible to selectively inhibit these types of BM-derived cells. The mechanism by which BM-derived cells contribute to tumor progression is complex; it involves incorporation of these cells into the tumor stroma and modulation of the tumor micro-environment. As the growth of most cancers is dependent on the interaction of tumor cells with their micro-environment, combined targeting of tumor growth-promoting BM-derived cells and other components of the tumor micro-environment may provide an efficient strategy to slow down tumor growth and improve the efficacy of cancer therapies. Alternatively, BM-derived cells, especially mesenchymal stem cells may be promising cellular vehicles for the selective delivery of cancer therapeutics. Results from pre-clinical studies in animals showed that this approach may be feasible; however, further research will be necessary regarding bio-safety issues and unwanted side effects before it can be tested in humans.

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Chapter 3

PTK787/ZK 222584 inhibits tumor growth promoting mesenchymal stem cells: kinase activity profiling as powerful tool in functional studies

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Abstract

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) have been shown to favour tumor growth, suggesting the relevance of pharmaceutical inhibition of MSCs for the treatment of malignancies. We tested the effect of PTK787/ZK 222584 (PTK) on the outgrowth of MSCs from human bone marrow-derived mononuclear cells (MNCs) and the migration and tube formation capacity of MSCs *in vitro*. PTK dose-dependently inhibited the outgrowth of BM-MSCs from BM-MNCs (LC50 1.12 μM PTK), while hematopoietic colony formation (HCF) was only slightly hampered (13 ± 19% at 1 μM PTK, and stable at ~50% at higher concentrations of PTK). Addition of 10 μM PTK inhibited proliferation of MSCs by 74 ± 6.6% compared to control ($p<0.0001$) and increased apoptosis of MSCs by 63 ± 7.7% ($p<0.01$). In addition, upon addition of PTK, BM-MSCs showed impaired tube formation as well as reduced migration (52 ± 19%, $p=0.006$) compared to control. Pepchip array analysis revealed that PTK effectively inhibits activity of kinases involved in cell cycling (WEE1 and several cyclin dependent kinases), and migratory processes (including Rho kinase). In conclusion, we show that PTK impairs outgrowth, proliferation, migration and tube formation of human BM-MSCs. In addition, we show the usability of Pepchip array analysis as a powerful tool for kinase activity profiling in functional studies since the effect of PTK on the kinome profile of MSCs corresponds with the observed functional effects of PTK on proliferation and migration. Inhibition of BM-MSCs and their contribution to tumor growth may be an additional strategy for treatment of cancer in the future.

Introduction

Recently, research is more and more focussed on the importance of the micro-environment, including bone marrow (BM)-derived mesenchymal stem cells (MSCs), for malignant progression. BM-derived hematopoietic stem cells and their progeny, such as VEGFR2-expressing endothelial progenitor cells (EPCs)(1), VEGFR1-expressing progenitor cells (niche-maker cells) (2) and Tie2-expressing monocytes (TEMs) (3), have been shown to contribute to tumor growth and progression. EPCs and TEMs are thought to promote tumor vessel formation by differentiation into endothelial cells or by the secretion of angiogenic factors respectively (1;3), while niche-maker cells are suggested to play a role in the initiation of metastatic niches (2). Recently, it was shown that non-hematopoietic MSCs also have the capacity to promote tumor growth (4-6). The identity, function and targeting of the various components of the micro-environment are only beginning to come to a sharper focus.
MSCs constitute a rare non-hematopoietic population in the adult bone marrow, and are progenitors of bone marrow stromal fibroblasts (7). MSCs are characterized by the expression of a large number of stromal cell markers (CD73, CD105, CD44, CD29, CD90) and the absence of hematopoietic (CD34, CD45, CD14) and endothelial markers (CD34, CD31, vWF) (7-9). MSCs express various cytokine and growth factor receptors and produce a large number of cytokines, growth factors and extracellular matrix proteins (fibronectin, vimentin, laminin) and have the potential to differentiate along osteogenic, adipogenic and chondrogenic lineages when placed in the appropriate environments (8;9).
It is hypothesized that MSCs can be mobilized into the peripheral blood. BM-derived MSCs have been detected in the myocardium after infarction in rats and mice (10;11). In humans, MSCs have been detected in peripheral blood from breast cancer patients after growth factor mobilization (12;13), as well as in non-mobilized blood from healthy subjects (14). Experimental evidence of fibroblast recruitment to tumor stroma in mice comes from the demonstration that intraperitoneously injected as well as intravenously injected human BM-derived β-gal-transduced MSCs preferentially localize within tumor stroma but not within the normal tissue stroma (15;16). In mice, it was observed that approximately 25% of αSMA-positive myofibroblasts and also some vimentin-expressing fibroblasts were BM-derived in

pancreatic tumors (17). BM-derived MSCs have been shown to favour tumor growth and progression of various xenografts in mice (4;6;18-20). BM-derived MSCs are thought to be involved in tumor new vessel formation(20) and the prevention of tumor cell recognition by the immune system (18). In addition, it has been hypothesized that MSCs promote the survival of cancer stem cells and protect these cells to drug-induced apoptosis (4).

These data indicate that BM-derived MSCs are not merely passive bystanders but are able to actively promote tumor growth and progression, suggesting the relevance of targeting BM-MSCs with pharmaceutical compounds for the treatment of malignancies. In this study we show that outgrowth, proliferation, migration capacity and tube formation capacity of human BM-MSCs *in vitro* is impaired by the small molecule tyrosine kinase inhibitor PTK787/ZK 222584 (21). Using Pepchip kinome profiling arrays, we show that PTK787/ZK 222584 inhibits activity of kinases involved in cell cycling (including WEE1 and several cyclin dependent kinases) and migration (including Rho kinase).

Materials and Methods

Culture of human BM-MNC - Bone marrow suspension cells were obtained from sternal marrow aspirations from donors who underwent thorax surgery, ranging in age from 26-75 years. All samples were obtained with written, informed consent. Mononuclear cells (MNCs) were isolated by density gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and washed with RPMI (Biowhitaker^{TM}, Verviers, Belgium). MNCs were plated in 24-well plates previously coated with 5 µg/ml fibronectin (Sigma Aldrich, Zwijndrecht, The Netherlands) for 2 hours at room temperature. Cells were plated at a concentration of $1x10^6$/ml and cultured in EBM2 supplemented with EGM2-MV SingleQuots with final serum concentrations of 5% (Cambrex, Clonetics, NY, USA) and 100 U/ml Pencilline and 100 µg/ml Streptomycin (Invitrogen, Gibco, Breda, The Netherlands). Cells were incubated in a 37ºC incubator with 5% CO_2 for 8 days. Fresh medium was added every 3rd or 4th day of culture. A growth curve was generated by passaging the cells every 3rd or 4th day of culture by trypsinization.

Characterization and counting of cultured adherent cells - After 8 days of culture adherent cells were washed with PBS. Cells were trypsinized with trypsine/EDTA for 10-15 min at 37°C, transferred into 4 ml tubes, centrifugated and resuspended in 500 µl PBS/EDTA. Reference beads were added and both cells and beads were counted on the FACS Calibur (BD Biosciences, Alphen aan de Rijn, The Netherlands). For characterization of cells, cell surface markers were evaluated by means of direct immuno-fluorescence. Cell suspensions (100 µl) were stained with 5 µl CD106-PE, CD105-FITC, CD90-APC, CD29-PE, CD146-PE, CD140b-PE, CD14-APC, CD45-PerCP, CD31-FITC, CD34-APC and VEGFR2-PE antibodies (BD Pharmingen, Alphen aan de Rijn, The Netherlands) for 20 min at RT. Cells were washed with PBS/EDTA and resuspended in 300 µl PBS/EDTA before FACS analysis. Data were analysed by the Winlist software (Verity Software).

Differentiation of cultured adherent cells into adipocytes and osteocytes - Adherent cells were tested for their ability to differentiate into adipocytes and osteocytes. To induce adipogenic differentiation, MSCs were cultured with 10^{-8} M dexamethasone and 5 µg/mL insulin (Sigma Aldrich, Zwijndrecht, The Netherlands). Oil-red-O (Sigma Aldrich, Zwijndrecht, The Netherlands) was used for staining of lipid droplets, characteristic for adipocytes. To induce osteogenic differentiation, MSCs were cultured in NH osteocyte differentiation medium (Miltenyi Biotec B.V., Utrecht, The Netherlands). 5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (SIGMA FAST BCIP/NBT substrate tablets; Sigma Aldrich, Zwijndrecht, The Netherlands) was used to detect expression of alkaline phosphotase (AP), an enzyme that is involved in the bone matrix mineralization, characteristic for osteocytes. Cells were incubated in a 37ºC incubator with 5% CO_2 for 14 days. Fresh medium was added every 3rd or 4th day of culture.

Administration of PTK787/ZK 222584 to MSC-cultures and CFU-assays - **MSC-cultures.** 1x10^6/ml BM-MNCs were plated in 24-well plates coated with fibronectin (Sigma Aldrich, Zwijndrecht, The Netherlands) and cultured in EBM2 supplemented with EGM2-MV SingleQuots (Cambrex, Clonetics, NY, USA) with 0, 1, 2.5, 5, 10, 20, 30 or 40 µM PTK787/ZK 222584 dissolved in DMSO (Merck, Darmstadt, Germany). DMSO was diluted (1:2000) in culturing medium and was also added to control wells

to correct for possible DSMO-induced cell death. Cells were incubated in a 37ºC incubator with 5% CO_2 for 8 days. Fresh medium supplemented with PTK787/ZK 222584 (a kind gift of the joint development project between Novartis Pharmaceuticals, Basel, Switzerland and Schering AG, Berlin, Germany) was added every 3rd or 4th day of culture. **CFU-assays.** $1x10^5$ BM-MNCs were added per ml CFU-GM semi-solid medium (R&D Systems, Abingdon, UK) supplemented with 0, 1, 5, 10, 20, 30, 40 and 50 µM PTK787/ZK 222584 dissolved in DMSO. DMSO was diluted (1:1000) in medium and was also added to control CFUs to correct for possible DSMO-induced cell death. Cells were plated in duplo in 6-well plates (Corning Inc Costar, NY, USA) and incubated in a 37ºC incubator with 5% CO_2 for 14 days. After 14 days colonies were counted by inverse microscopy.

Proliferation assay - Proliferation of MSCs derived from 4 different donors was assessed using the BrdU Cell Proliferation Assay (CalBiochem, Merck, Nottingham, UK) according to the protocol provided. MSCs were seeded at a density of 10.000 cells/well in a 96-well plate and incubated with PTK787/ZK 222584 for 48 hours before adding the BrdU label for another 16 hours. Experiments were performed in quadruple.

Apoptosis assay - Apoptosis of MSCs derived from 4 different donors was assessed using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the protocol provided. MSCs were seeded at a density of 300.000 cells/well in a 6-well plate and incubated with PTK787/ZK 222584 for 48 hours. Cells were trypsinised, centrifuged and stained with Annexin-V-FITC/PI and analysed using FACS Calibur and Winlist software.

Migration assay - Transwells were prepared by adding 30 µl Biocoat Matrigel (BD Biosciences, Alphen aan de Rijn, The Netherlands) onto the membrane of 24 wells transmigration chambers (Costar, Corning Inc, NY, USA) and incubating the transwells overnight at room temperature. The lower wells were filled with 500 µl EBM2 with 5% FCS, supplemented with EGM2-MV SingleQuots (Cambrex, Clonetics, NY, USA). Matrigel coated membranes were then overlaid with 5×10^4 MSCs suspended in 50 µl EBM2 (Cambrex, Clonetics, NY, USA) with 1% FCS. One

µM PTK787/ZK 222584 was added to the medium in the upper or lower wells. Transwells were incubated overnight in a 37ºC incubator with 5% CO_2. The next day, after removal of the Matrigel, membranes were fixed in methanol for 5 minutes, stained with Giemsa solution (Sigma Aldrich, Zwijndrecht, The Netherlands) for 5 minutes and mounted on a slide. Migrated cells were counted in 3 high power fields (400x) using a microscope with a digital camera Olympus BX50. MSCs from 3 different donors were used.

Tube formation assay - Collagen gels were prepared by adding 120 µl Biocoat Matrigel (BD Biosciences, Alphen aan de Rijn, The Netherlands) to a polystyrene 4-chamber culture slide (BD Falcon, Alphen aan de Rijn, The Netherlands). Slides were incubated in a 37ºC incubator with 5% CO_2 for 30 minutes. Gels were then overlaid with 5 x 10^4 viable cells suspended in 500 µl EBM2 supplemented with EGM2-MV SingleQuots (Cambrex, Clonetics, NY, USA). Slides were incubated in a 37ºC incubator with 5% CO_2. After 2, 4 and 6 hours of incubation, progression of tube formation was determined using a phase-contrast microscope with a digital camera Olympus BX50. MSCs treated with 2 µM PTK787/ZK 222584 were used to compare with DMSO-treated control MSCs. MSCs from 2 different donors were used. HUVEC and HL60 were used as positive and negative control, respectively. Viability of the cells was checked with Trypan blue staining right before starting the assay. The number of viable cells incubated on the Matrigel was similar in the treated and control group.

RNA isolation and RT-PCR - After 8 days of culture RNA was isolated from adherent cells, using Machery Nagel RNA isolation kit (Machery Nagel, Düren, Germany), according to the protocol provided. cDNA were prepared by reverse transcription at 37°C for at least 1 h in a 20-µl reaction mixture containing 2,5 µg of total RNA, random hexamers (Pharmacia), 5x first-strand buffer, RNasin, and 2 µl of reverse transcriptase (Life Technologies, Inc.). cDNA was amplified in the presence of primers, 10x buffer, 1.5 mM $MgCl_2$, deoxynucleotide triphosphates, and Taq (Life Technologies, Inc.). The mixture was amplified in a Perkin-Elmer apparatus with PCR cycle conditions specific for the PCRs tested. The PCR product was analyzed by electrophoresis in a 1.5% agarose gel. Gels were stained with ethidium bromide and

photographed. Gene expression from PDGFR, EGFR, FGFR, VEGFR3, VEGFR2, and VEGFR1 was assessed by using the following primer combinations (Invitrogen, Breda, The Netherlands). PDGFR forward TGACCACCCAGCCATCCTTC, reverse GAGGAGGTGTTGACTTCATTC (228 BP); EGFR forward: ACCAGAGTGATGTCTGGAGC, reverse GATGAGGTACTCGTC-GGCAT (377 BP); FGFR forward AAGGACAAACCCAACCGTGTGACC, reverse GCCAAAGTCTGCTATCTTCATCAC (426 BP); VEGFR3 forward CAAGCCATCCGA-GGAGCTAC, reverse GTCTTGCACTTCGCACACATAGTGG (285 BP); VEGFR2 forward ATGACATTTTGATCATGGAGC, reverse CCCAGATGCCGTGCATGAG (211 BP); VEGFR1 forward GGAAGGCATGAGGATGAGAG, reverse CAGAGAAGGCAGGAGTTGAG (161 BP). PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. HUVECs were used as positive controls.

PepChip kinome profiling arrays - For kinome array samples, 10^6 MSCs obtained from two different donors were pre-incubated with 1 µM PTK for 1h and lysed in 100 µl of cell lysis buffer (Mammalian Protein Extraction Reagent (M-PER) with HALT protease inhibitor cocktail (1:100) and HALT phosphatase inhibitor cocktail (1:100) without EDTA (all from Pierce Biotechnology, Etten-Leur, The Netherlands). The cell lysates were spun down for 10 min at 4 °C and 80 µl supernatant was used for kinome analysis. Peptide array incubation mix was produced by adding 20 µl of filter-cleared activation mix (50% glycerol, 70 mM $MgCl_2$, 70 mM $MnCl_2$, [γ-^{33}P]ATP (1000 kBq)). PTK787/ZK 222584 was added to the peptide array mix to a final concentration of 10 µM. DMSO was added to the control peptide array mix to correct for the solvent. The peptide array mix was added onto the Pepchip, on which three identical arrays are spotted (Pepscan, Lelystad, The Netherlands). The chip was kept at 37 °C in a humidified stove for 90 min, washed in 2 M NaCl, in PBS with 1% SDS, and in demineralized H_2O and air-dried. The Pepchips were exposed to a phospho-imager screen for 72 h (Fuji, Stamford, CO). The full list of the peptides spotted on the peptide array can be found online (http://www.pepscan.nl/pdf/Content%20PepChip%20Kinase%20%200303.pdf).
After data acquisition using the phospho-imager, spot densities were quantified with Scanalyze software and exported to Microsoft Excel. Spot densities were corrected for the individual background to diminish interarray variances. The variation between

arrays was reduced by normalization to the 99th percentile of the intensity of each array. Spot densities were ranked by a signal intensity score ranging from 0 to 100. Inconsistent data (i.e. SD between the data points > 1.96 of the mean value) were excluded from further analysis. The average spot density for each peptide was calculated using data from triplicate experiments of both MSC-lysates. Differential kinase activities in lysates from cells incubated with or without PTK787/ZK 222584 were determined using the following analysis restrictions: mean signal intensity 0μMPTK > threshold value of 10 AND mean signal intensity 0μMPTK > mean signal intensity 10μMPTK AND mean signal intensity 0μMPTK > mean signal intensity 100μMPTK AND mean signal intensity ratio of 10μMPTK and 100μMPTK versus 0μMPTK > 1 standard deviation from the mean. The Phospho-ELM BLAST tool within the Phosphobase resource (release 7.0) was applied to determine the kinases potentially responsible for phosphorylation of a specific peptide (http://phospho.elm.eu.org/pELMBlastSearch.html).

Western blot - PTK-treated and untreated MSCs were lysed in RIPA lysis buffer + Complete™ solution (Roche Diagnostics GmbH, Penzberg, Germany) and 1 mM Na_3VO_4. Laemmli sample buffer was added and the lysate was boiled for 5 minutes. Proteins were separated using a 10% SDS/polyacrylamide gel, blotted onto a PVDF membrane (Millipore, Etten-Leur, the Netherlands), blocked with Tris-buffered saline + 1% Tween-20 and 5% Bovine Serum Albumine (Sigma Aldrich, Zwijndrecht, The Netherlands) (TBST/BSA). Blots were incubated overnight at RT in TBST/BSA containing a primary antibody (anti-FAK, anti-pFAK(Tyr397) (BD Biosciences, USA), anti-ERK, anti-pERK(Thr202/Tyr204), anti-CDC2, anti-pCDC2(Tyr15) (Cell Signaling, USA) (all 1:2000), followed by incubation with the appropriate secondary peroxidase-conjugated antibodies (Dako, Denmark) (1:5000) at RT for one hour. Antibody binding was visualized by enhanced chemoluminescence. β-actin was probed as a protein loading control (1:3000; sc-47778, Santa Cruz Biotechnology, USA).

Statistical analysis - All values in the figures represent mean ± SD. The relevant data sets were compared by unpaired Student's t test using SPSS software. P-values <0.05 were considered statistically significant.

Results

Phenotype of cultured human BM-derived cells.

Mononuclear cells isolated from human bone marrow aspirates, were cultured in EBM2 to assess mesenchymal stem cell properties under angiogenic conditions, resembling angiogenic tumor micro-environments. Small adherent colonies appeared at day 3-4, and fibroblast-like cells appeared around these colonies. These initially small colonies expanded to larger colonies at day 7-8 and by then, the culture plate was covered with a confluent layer of fibroblast-like cells. In the centre of these colonies cobblestone-like cells were observed. Cultured adherent cells could be expanded for up to 7 passages. After the sixth passage, proliferation ceased and some cells spontaneously differentiated into adipocytes or osteocytes. Cultured adherent cells were positive for CD90, CD29, CD106, CD140b, CD146, and CD105, and negative for CD45, CD34, CD14 and CD31 (figure 1A), which is typical for mesenchymal stem cells (MSCs). Upon stimulation, cultured adherent cells were able to differentiate into adipocytes and osteocytes (figure 1B).

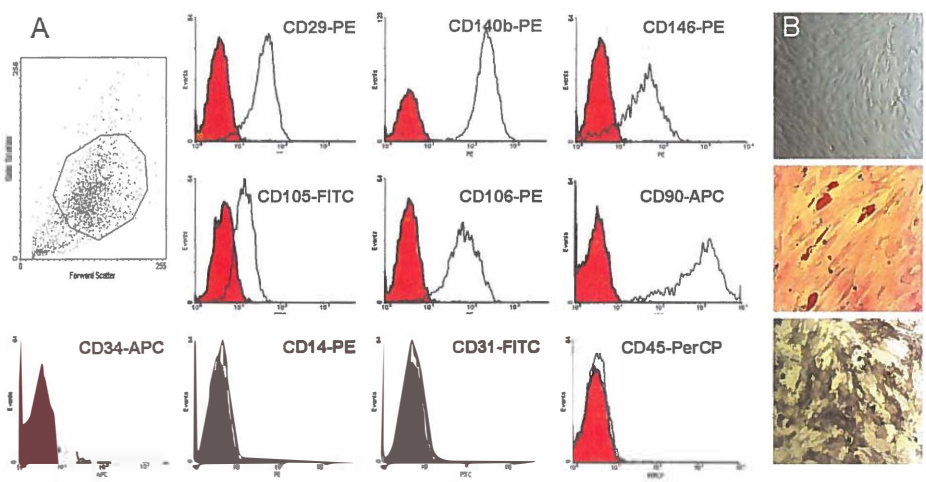

Figure 1: Phenotype of cultured adherent fibroblast-like cells by flow cytometry. *A) Histograms for indicated antigens; open curves represent protein expression, filled curves represent isotype-matched antibody controls; B) Adherent cells after culture in control medium (top panel), adipocyte differentiation medium (middle panel), and osteocyte differentiation medium (lower panel) (100x). Adipocytes are characterized by intracellular lipid droplets, stained red by Oil red O. Osteocytes were stained purple/brown representing alkaline phosphotase (AP)-activity.*

PTK787/ZK 222584 reduces the outgrowth of MSCs from BM-MNCs.

With the knowledge that MSCs can contribute to tumor growth, we assessed whether the outgrowth of MSCs from human BM-MNCs could be inhibited by PTK787/ZK 222584. Administration of PTK787/ZK 222584 led to a dose dependent decrease of mesenchymal outgrowth from human BM-MNCs (figure 2). In primary cultures supplemented with 1 µM PTK787/ZK 222584, the outgrowth of mesenchymal cells was significantly decreased compared to control without PTK (median cell number: 20.184 [57.045-1.485] and 53.651 [83.093-11.457] respectively, p=0.0015). LC50, defined as the concentration of PTK787/ZK 222584 at which the number of MSCs was reduced by 50% compared to control, was 1.12 µM. Mesenchymal outgrowth was completely blocked at a PTK787/ZK 222584 concentration of 20 µM or higher. Sporadically some fibroblast-like cells, but no colonies could be detected in these wells by light microscopy.

To determine whether administration of PTK787/ZK 222584 would affect hematopoietic colony formation as a possible negative side-effect of *in vivo* use, CFU-GM assays were performed. 1 µM PTK787/ZK 222584 did not affect colony formation compared to control without PTK (median colony number: 182 [78-227] and 180 [103-255] respectively, p=0.38). At higher concentrations of PTK787/ZK 222584, colony formation remained stable at a level of ~50% of control (median colony number at 20 µM: 91 [55-139]). The numbers of granulocytic and myelocytic colonies were equally distributed (data not shown).

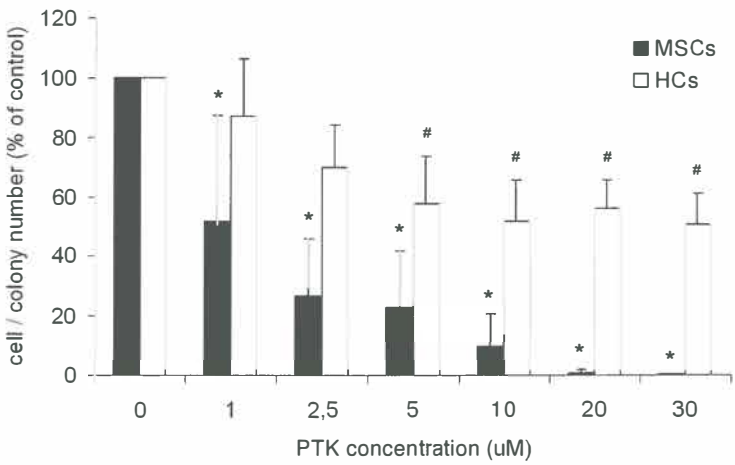

***Figure 2**: The effect of PTK787/ZK 222584 on the outgrowth of mesenchymal stem cells (MSCs) from human bone marrow mononuclear cells (BM-MNCs) and the formation of hematopoietic colonies (HCs). Fresh BM-MNCs were obtained from 16 healthy individuals and incubated with PTK787/ZK 222584 in liquid cultures for 8 days (MSCs) or in a CFU-assay for 14 days (HCs). Control wells were incubated with DMSO to correct for the solvent. Each bar represents the mean percentage ± stdev of cultured adherent MSCs compared to its DMSO-control (black bars; *p<0.01) or the mean percentage ± stdev of hematopoietic colonies compared to its DMSO-control (white bars; #p<0.05).*

PTK787/ZK 222548 inhibits proliferation and induces apoptosis of MSCs.

Next, we tested the effect of PTK787/ZK 222584 on proliferation of MSCs by measuring BrdU incorporation into the DNA of proliferating cells. Addition of 1 μM PTK787/ZK 222584 significantly inhibited proliferation of MSCs by 12 ± 6.5% compared to control without PTK (p=0.015) (figure 3A). Addition of 10 μM PTK787/ZK 222584 resulted in a reduction of 74 ± 6.6% compared to control (p<0.0001) and 50 μM PTK787/ZK 222584 almost completely blocked proliferation of MSCs (91 ± 1.2% reduction compared to control, p<0.0001).

In addition, we investigated the effect of PTK787/ZK 222584 on apoptosis of MSCs. Addition of 1 μM PTK787/ZK 222584 significantly increased the percentage of Annexin-V/PI double-positive apoptotic MSCs by 49 ± 3.1% compared to control without PTK (mean percentage of apoptotic cells: 26 ± 7.5% and 18 ± 5.1% respectively, p=0.037) (figure 3B). The percentage of apoptotic MSCs was increased by 63 ± 7.7% after addition of 10 μM PTK787/ZK 222584 and by 131 ± 31% after addition of 50 μM PTK787/ZK 222584 compared to control (p<0.01). These results indicate that PTK787/ZK 222584 inhibits growth of MSCs by inhibiting proliferation and inducing apoptosis.

PTK787/ZK 222584 impairs migration capacity of MSCs.

To study the migration capacity of MSCs in a PTK787/ZK 222584-rich environment, we performed migration assays. We show that the migration capacity of MSCs is inhibited by PTK787/ZK 222584 (figure 3C). Administration of 1 μM PTK787/ZK 222584 to the cell suspension in the upper well resulted in 52 ± 19% reduction of migrated MSCs compared to control without PTK (mean number of migrated cells: 47 ± 19 and 108 ± 5 respectively, p=0.006). In addition, administration of PTK787/ZK 222584 to the lower well also inhibited migration of MSCs compared to DMSO-

control (mean number of migrated cells: 27 ± 19, p=0.002), suggesting that MSCs are functionally impaired in a PTK787/ZK 222584-rich environment.

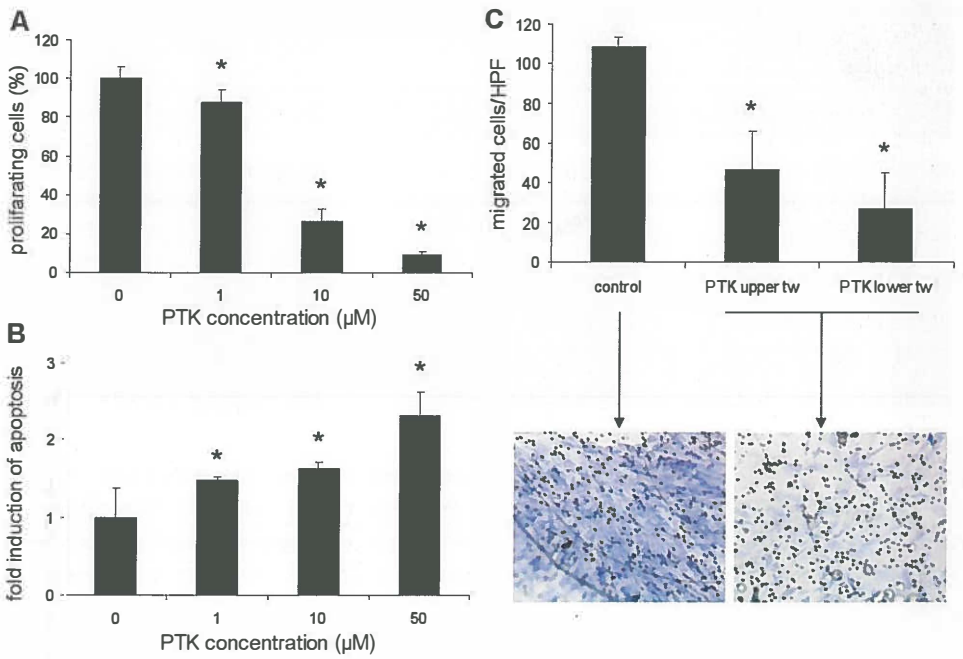

Figure 3: The effect of PTK787/ZK 222584 on proliferation, apoptosis and migration of MSCs. *A) Percentage of proliferating cells in MSC-cultures incubated with PTK787/ZK 222584 at indicated concentrations compared to DMSO-control. *p<0.05. B) Fold increase of the percentage of apoptotic MSCs induced by PTK787/ZK 222584 at indicated concentrations compared to DMSO-control. *p<0.05. C) Number of migrated MSCs after addition of 1 μM PTK787/ZK 222584 to the cell suspension in the upper transwell (tw) or to the medium in the lower transwell compared to DMSO-control. *p<0.01. Lower panel: examples of Giemsa staining of MSCs that have migrated through the membrane pores.*

PTK787/ZK 222584 impairs tube formation capacity of MSCs.

Tube formation properties of MSCs were studied using tube formation assays in Matrigel. After 2h of incubation on collagen gel, MSCs started to from tube-like structures and made some connections with neighbouring cells. After 6h the tube-like network was as extensive as the positive control (HUVEC). Negative control (HL60 cells) did not show any tube formation. Interestingly, MSCs treated with 2 μM PTK787/ZK 222584, showed reduced tube formation (figure 4). These data indicate that PTK787/ZK 222584 inhibits tube formation capacity of MSCs *in vitro*.

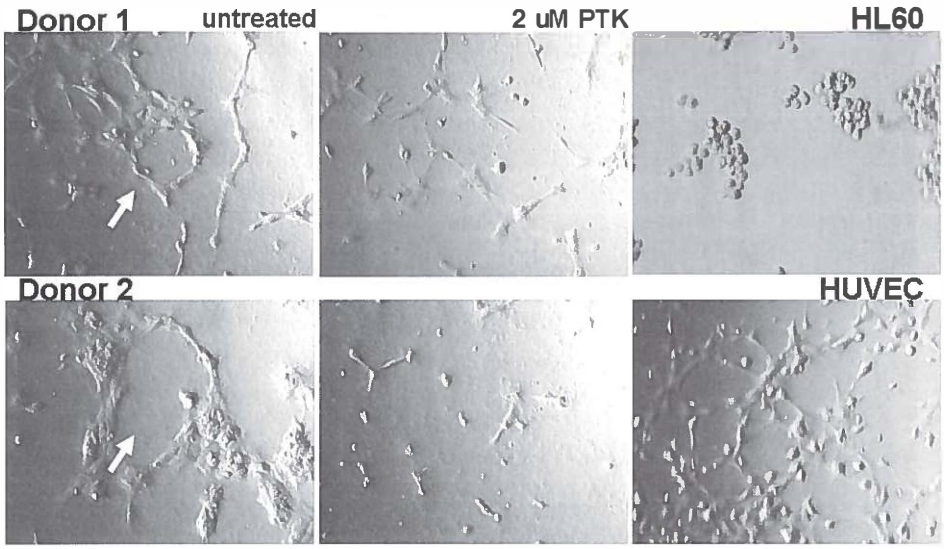

Figure 4: PTK787/ZK 222584 inhibits tube formation properties of MSCs. *Pictures show tube formation of untreated MSCs (left), MSCs treated with 2 µM PTK787/ZK 222584 (middle), HL60-cells (negative control) and HUVECs (positive control) (right). Pictures were taken after incubation of the cells on Matrigel for 4 hours. Arrows indicate completely formed tubes, which were absent in Matrigels with PTK787/ZK 222584-treated MSCs. MSCs of two different donors were used.*

PTK787/ZK 222584 inhibits the activity of kinases involved in cell cycling and migratory processes. It is known that PTK787/ZK 222584 inhibits kinase activity of VEGF-receptors and PDGFR in the nanomolar range, and does not inhibit kinase activity of EGFR or FGFR in concentrations up to 10 µM (21;22). Using RT-PCR, we found that MSCs expressed PDGFR, EGFR and FGFR, but lack expression of VEGF-receptors (figure 5A), suggesting that, in MSCs, PTK787/ZK 222584 would mainly interfere with signalling through PDGFR. To study the effect of PTK787/ZK 222584 on the activity of kinases in MSCs we used Pepchip kinome profiling arrays (23;24). Current mass spectrometry techniques and novel proteomics approaches like antibody microarrays determine protein phosphorylation levels rather than the enzymatic activity resulting from it, while measurement of kinase activity using the peptide microarray provides a direct view on the extent of enzymatic activity leading to specific signal transduction.

Data analysis resulted in a list of 53 peptides that showed decreased phosphorylation upon administration of PTK787/ZK 222584 compared to control arrays (table 1). With the aid of the Phospho-ELM database, potential upstream kinases responsible for the phosphorylation of the found peptides were identified. Phosphorylation of 8 peptides (Vimentin, Stomatin, Fgr, ATP2B1, EphB1, CDK1, PTEN, and BCL2-interacting protein BIM) was significantly decreased in both the 10 µM and the 100 µM PTK787/ZK 222584 arrays compared to control ($p<0.05$, figure 5B). The potential upstream kinases for these peptides are Rho, PKA, Fgr, PKC, EphB1, WEE1/CDK7, CK2, and JNK1, respectively. Interestingly, WEE1 is a key kinase coordinating the transition between DNA replication and mitosis (25). CDK7 and CK2 are other kinases involved in cell cycling processes (26;27). In addition, Rho is a key kinase regulating cellular migration (28).

As depicted in table 1, the potential upstream kinases of 15 peptides (WEE1, CK2, ATM, CDK5, CDK7, CDK5, CDK1, CDK2, and ATR) were classified to signal transduction pathways regulating cell cycling processes. The potential upstream kinases of 5 peptides (Fgf, Rho, Fyn, and FAK) were classified to signal transduction pathways regulating cellular migration. The potential upstream kinases of 9 peptides (EphB1, FGFR2, Bruton'sTK, c-Met, EphB2, EGFR, and ABL) were classified to receptor signalling. Decreased peptide-phosphorylation of peptides derived from the PDGF-receptor (4 peptides present on the pepchip) was not observed. In addition, we found some very common potential upstream kinases, such as PKC, PKA, AKT, and several members of the MAP kinase family to be down-modulated upon administration of PTK787/ZK 222584.

To confirm the data from the kinase array, we performed western blotting followed by immunodetection with phospho-specific antibodies. Decreased levels of phosphorylated ERK1/2 (MAP kinase), and CDC2 (cell cycling) could be detected in MSCs incubated with 10 µM PTK787/ZK 222584. In addition, decreased levels of phosphorylated FAK (migration) were detected in MSCs incubated with 100 µM PTK787/ZK 222584.

In conclusion, these data show that, in MSCs, PTK787/ZK 222584 inhibits activity of kinases involved in cell cycling and migration processes. The effect of PTK787/ZK 222584 on the kinase activity profile, thus, nicely corresponds with the observed inhibitory effects of PTK787/ZK 222584 on proliferation and migration.

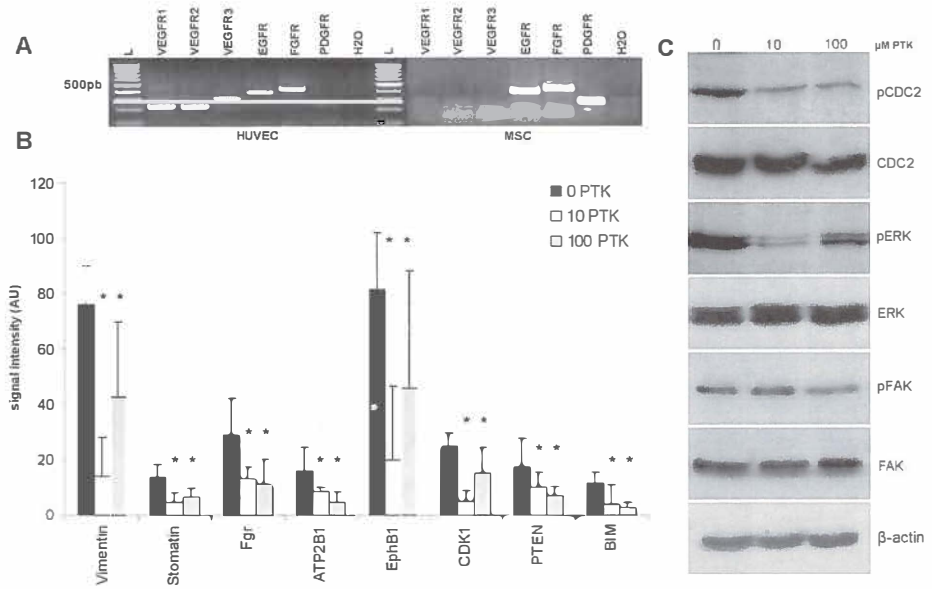

Figure 5: RT-PCR, Pepchip Array, Western blotting. *A) PCR products for indicated tyrosine kinase receptors in MSCs. HUVEC was used as positive control. B) PTK787/ZK 222584 down-modulates phosphorylation levels of indicated peptides as assessed by Pepchip kinome profiling arrays. Y-axis represents the level of peptide phosphorylation (signal intensity). Bars represent mean signal intensities of array triplicates from MSC-lysates derived from 2 different donors (arbitrary units ± stdev). Black bars: DMSO-control; white bars: MSCs incubated with 10 µM PTK787/ZK 222584 for 1 h; grey bars: MSCs incubated with 100 µM PTK787/ZK 222584 for 1 h. *p<0.05 C) Western blots of MSCs incubated with 10 µM or 100 µM PTK787/ZK 222584 compared to DMSO-control. Levels of phosphorylated proteins were determined using phospho-specific antibodies.*

Table 1 (next pages): The effect of PTK787/ZK 222584 on the kinome profile of MSCs. *PTK787/ZK 222584 was added to the MSC culture for 1h right before making the lysates. PROT_ID: protein identification number; MOTIF: peptide-sequence present on the array; PROTEIN: protein from which the peptide-sequence is derived; UP-KIN: potential upstream kinases annotated by the Phospho-ELM database; 0-10-100 µM PTK: mean signal intensity (arbitrary units) and standard deviations from DMSO control, 10 µM PTK, 100 µM PTK arrays, respectively; ratio 10/0 and ratio 100/0: signal intensity from the 10 µM PTK and 100 µM PTK arrays, respectively, divided by signal intensity from the DMSO control; t-test 0vs10 p-values: 10 µM PTK versus DMSO control; t-test 0vs100 p-values: 100 µM PTK versus DMSO control. Experiments were performed in triplicate using MSCs from 2 different donors. Potential upstream kinases are classified by the cellular process they contribute to (cell cycle, migration, receptor and intracellular).*

Prot-ID	MOTIF	PROTEIN	Upstream Kinase	0 µM PTK		10 µM PTK		100 µM PTK				t-test 0vs10	t-test 0vs100
				mean	SD	mean	SD	mean	SD	ratio 10/0	ratio 100/0	p value	p value
			CELL CYCLE										
NP_001777	PIRVYTHEVVT	CDK1	WEE1/CDK7	24,9	4,9	18,5	4,1	15,3	9,4	0,7	0,6	0,0231	0,0254
NP_000305	SSTSVTPDVSD	PTEN	CK2	17.7	10,3	7,1	5,4	7,2	3,4	0,4	0,4	0,0341	0,0284
NP_005554	PEFPLSPPKKK	Stathmin 1	CDK1	38,0	21,0	17,9	12,0	19,6	19,1	0,5	0,5	0,0346	0,0721
NP_005648	DSQPESQVLED	Tumor protein p53 binding protein 1	ATM	37,0	24,6	13,9	4,8	20,7	9,0	0,4	0,6	0,0356	0,0799
NP_003370	KDDKLTPKIGF	Eznn	CDK5	58,5	21,8	36,2	25,3	23,6	16,1	0,6	0,4	0,0667	0,0052
NP_002469	GDSDASSPRSN	MyoD	CDK1	13,1	6,5	7,1	6,4	8,1	5,8	0,5	0,6	0,0701	0,0960
NP_009225	GVERSSPSKCP	BRCA1	CDK2	19,5	14,3	9,5	6,4	2,4	2,5	0,5	0,1	0,0739	0,0082
NP_110435	ENTFPSPKAIP	Nude like protein	CDK5	44,4	31,1	23,5	10,7	22,8	11,4	0,5	0,5	0,0937	0,0708
NP_066566	APRQSSPSKSS	DAB1	CDK5	21,2	20,9	9,9	8,4	5,1	4,0	0,5	0,2	0,1241	0,0635
NP_005564	PTTPLSPTRLS	Lamin B1	CDK1	67,1	23,2	50,2	25,1	47,6	25,4	0,7	0,7	0,1265	0,0978
NP_000012	GPHRSTPESRA	Presenilin 1	CDK5	17,4	17,6	8,1	7,0	7,8	6,2	0,5	0,4	0,1302	0,1196
NP_001265	RPRVTSGGVSE	Cell cycle checkpoint kinase I	ATR	22,5	28,8	7,6	3,2	6,4	1,6	0,3	0,3	0,1415	0,1231
NP_005162	QVSSLSESEES	Activating transcription factor 1	CK2	20,4	17,8	13,0	5,3	12,0	10,1	0,6	0,6	0,1772	0,1718
NP_001777	KIGEGTYGVVY	CDK1	WEE1/CDK7	22,4	16,6	14,9	4,1	13,9	7,4	0,7	0,6	0,1805	0,1609
NP_002859	LQRQASPSIVI	Ras associated protein Rab5B	CDK1	11,5	12,0	6,4	6,9	6,3	4,4	0,6	0,5	0,1956	0,1723
			RECEPTOR										
NP_004432	PGMKIYIDPFT	EphB1	EphB1	81,7	20,1	49,8	26,7	45,8	42,4	0,6	0,6	0,0207	0,0451
NP_075261	TTNEEYLDLSQ	Fibroblast growth factor receptor 2	FGFR2	34,1	15,2	20,8	6,4	25,4	8,3	0,6	0,7	0,0518	0,1222
AAH11772	NQLFLYDTHQN	Phospholipase C, gamma 2	Bruton's TK	28,2	10,1	19,4	7,2	13,3	10,4	0,7	0,5	0,0564	0,0149
NP_000236	YDKEYYSVHNK	Hepatocyte growth factor receptor	c-Met	48,9	32,0	25,1	19,7	27,2	16,7	0,5	0,6	0,0758	0,0863
NP_002491	FDGPLSPPLSI	Neurogenic differentiation factor 1	EphB2	28,6	23,5	14,8	10,8	12,8	6,7	0,5	0,4	0,1093	0,0715
AAD42222	DGENIYIRHSS	Erythrocyte membrane protein band 41	EGFR	20,8	18,1	12,2	5,5	10,8	9,8	0,6	0,5	0,1459	0,1306
NP_001761	MRGILYAAPQL	CD19	ABL	23,1	23,8	11,8	10,2	10,2	7,2	0,5	0,4	0,1566	0,1172
NP_004439	AENPEYLGLDV	ErbB2	ErbB2, EGFR	38,4	17,9	28,7	13,7	21,8	14,3	0,7	0,6	0,1597	0,0536
NP_005689	ATLDVYNPFET	SCAMP3	EGFR	31,3	14,2	23,1	15,3	22,7	8,4	0,7	0,7	0,1809	0,1332

Prot-ID	MOTIF	PROTEIN	Upstream Kinase	0 µM PTK		10 µM PTK		100 µM PTK				t-test 0vs10	t-test 0vs100
				mean	SD	mean	SD	mean	SD	ratio 10/0	ratio 100/0	p value	p value
			MIGRATION										
NP_003371	AVRLRSSVPGV	Vimentin	Rho Kinase	75,9	14,2	40,6	13,9	42,7	26,9	0,5	0,6	0,0007	**0,0117**
NP_005239	IKDDEYNPCQG	Fgr	Fgr	28.9	13,4	10,4	4,3	11,3	9,0	0,4	0,4	0,0083	**0.0121**
NP_001456	DDQEVYDDVAE	FYB	Fyn	25,3	18,6	14,4	8,8	14,3	7,6	0,6	0,6	0,1124	0,1046
BAA18998	EEEHVYSFPNK	Paxillin	FAK	69.5	21.4	52.1	25,5	49,5	22.2	0,7	0,7	0,1192	0.0744
NP_003028	PCTTIYVAATE	SLAM	Fyn	25.0	14,4	18,7	7.8	13,4	9,7	0,7	0,5	0,1819	0,0655
			INTRACELLULAR										
NP_004090	RDTRDSEAQRL	Stomatin	PKA	13.6	4,8	4,3	3,4	6,7	3,2	0,3	0,5	**0,0015**	**0,0074**
NP_001673	LNRIQTQIRVV	ATP2B1	PKC	16,2	6,5	8,0	1,8	4.7	3,7	0,5	0,3	**0.0206**	**0,0061**
NP_619527	STQTPSPPCQA	BCL2-interacting protein BIM	MAPK8	11,7	3,8	4,9	7,3	2.9	1,9	0,4	0,2	**0,0365**	**0,0006**
NP_001612	NGRPDYIIVTQ	Aryl hydrocarbon receptor	-	22,3	10,3	11,6	7,4	16,0	6,9	0,5	0,7	**0,0416**	0,1190
NP_892113	PDHQYYNDFPG	Shc	SYK, c-Src	22,8	12,3	11,9	7,4	14,1	1,6	0,5	0,6	**0,0463**	0,0580
NP_002944	EFTSRTPKDSP	Ribosomal S6 kinase 1	MAPK1	24,9	14,1	12,6	9,0	9,0	4,7	0,5	0,4	0,0517	**0.0129**
NP_444284	LDRFLSLEPVK	Cyclin D1	PKA	68,2	11,7	49,2	23,5	47,7	21,4	0,7	0,7	0,0540	**0,0332**
BAA18998	LPGALSPLYGV	Paxillin	MAPK8	49.5	23,1	29,6	16,4	37,1	28,4	0,6	0,7	0,0589	0,2449
NP_006194	PFRRHSWICFD	cAMP specific 3',5 '-cyclic phosphodiesterase 4D	PKA	13,8	6,0	7,8	6,3	10,1	5,3	0,6	0,7	0,0687	0,1370
P15941	VPPSSTDRSPY	Mucin 1 transmembrane	PKC-delta	50,4	22,8	30,5	23,2	20,8	8,3	0,6	0,4	0,0819	**0,0116**
NP_006248	TTVELYSLAER	PKC- theta	Lck	20,1	8,4	12,3	10,8	13,6	4,9	0,6	0,7	0,0954	0,0829
NP_002960	MSSPPPARSG	MAPK12	MAPK12	14.1	6,3	9,1	4.7	4.8	3,4	0,6	0,3	0,1137	**0,0150**
NP_005456	ERMNCSPTSQI	AKT3	PDK1	21.0	28,5	5,4	8,7	3,4	3,3	0,3	0,2	0,1138	0,1035
NP_001952	GETRFTDTRKD	Eukaryotic translation elongation factor 2	eEF2 Kinase	32,7	15,0	22,7	13,0	16,3	4,0	0,7	0,5	0,1229	**0,0219**
NP_127492	PFRRPSTYGIP	TFII-I	PRKG1	19.5	14,7	10,7	9,8	4,8	5,4	0,5	0,2	0,1238	**0,0320**
NP_005154	GATMKTFCGTP	AKT1	PDK1	16.4	8,4	10,9	7.7	6.0	6,4	0,7	0,4	0,1322	**0,0183**
NP_001058	AEVLPSPRGQR	DNA topoisomerase II alpha	MAPK	69,0	19,0	51,4	32,1	49,2	16,6	0,7	0,7	0,1375	**0,0414**
NP_000336	PSEEGYQDYEP	Synuclein alpha	SYK	24,2	10,5	18,1	9,6	14,4	11,0	0,7	0,6	0,1609	0,0738
NP_003143	LDSRLSPPAGL	STAT5A	MAPK	17,3	13,3	10,2	10,2	12,4	7,1	0,6	0,7	0,1640	0,2247
NP_061485	RLRPLSYPQTV	Ras related C3 botulinum toxin substrate 1	AKT1	32.4	32,8	17,1	11,1	16,1	8,1	0,5	0,5	0,1738	0,1546
NP_002388	LPSTQSLNIKS	Myocyte specific enhancer factor 2C	MAPK7	21,6	16,3	14,0	10,5	13,4	10,7	0,6	0,6	0,1796	0,1643
NP_004324	RDRSSSAPNVH	b-Raf	AKT, SGK	59.5	31,7	43,4	27,0	43,7	22,3	0,7	0,7	0,1825	0,1711
NP_002739	HKGHLSEGLVT	MAPK6	MAPK6	28,4	30,9	16,4	8,4	10,0	8,3	0,6	0,4	0,1898	0,1158

Discussion

A growing body of evidence indicates the importance of the micro-environment, including BM-MSCs, for malignant progression. Research has indicated that 1) BM-derived MSCs can be present in the peripheral blood (12-14); 2) circulating MSCs are able to migrate towards tumors and incorporate into the tumor stroma (5;16); 3) MSCs can contribute to tumor growth and progression (4;6;19;20). These findings indicate that MSCs are a potential pharmacological target for anti-cancer therapies. In this study, we show that PTK787/ZK 222584 inhibits outgrowth, proliferation and migration of MSCs, impairs the tube formation capacity of MSCs, and induces apoptosis of MSCs. Using Pepchip kinome profiling arrays, we show that PTK787/ZK 222584 inhibits the activity of kinases involved in cell cycling and migration processes, results that were verified by western blotting and correspond with the observed functional effects of PTK787/ZK 222584 on proliferation and migration of MSCs.

MSCs are hypothesized to contribute to tumor vessel formation by the production of pro-angiogenic cytokines and by endothelial trans-differentiation of MSCs (6;29). In accordance with other studies (29;30), we showed that MSCs have the pro-angiogenic capacity to spontaneously form capillary (tube)-like structures *in vitro*. We observed that PTK787/ZK 222584 impaired the tube formation capacity of MSCs, suggesting that PTK787/ZK 222584 may partly prevent pro-angiogenic activity of MSCs. Endothelial trans-differentiation of MSCs was not observed in our angiogenic culturing system, since MSCs still lacked expression of endothelial specific markers CD34, CD31 or VEGFR after 8 days of culturing. Prolonged culturing of MSCs in angiogenic medium for at least four weeks has been shown to result in differentiation of MSCs into endothelial-like cells expressing VEGFR2 (30) or CD34 (29), indicating that eight days of culturing in our model is too short to prove endothelial trans-differentiation of MSCs.

It is known that PTK787/ZK 222584 inhibits kinase activity of VEGFR1, 2 and 3, c-kit, c-fms, PDGFR in the nanomolar range, and does not inhibit kinase activity of EGFR or FGFR for up to 10 μM (21;22). In accordance with results from Satomura et al. (31), we showed that MSCs lack expression of VEGF-receptors, but do express PDGFR, EGFR and FGFR. Ligands for these receptors (PDGF, EGF, and FGF,

respectively) are known to be important growth factors for MSCs (32;33). Ligand binding to membrane receptors results in activation of receptor-kinases that in their turn initiate signalling pathways, such as the MAP kinase pathway. Signal transduction through these pathways eventually can result in a number of biological processes, including cellular migration and cell cycling (33). Interestingly, using Pepchip array analysis, we found that PTK787/ZK 222584 inhibits peptide-phosphorylation of potential upstream kinases that could be classified by their involvement in signal transduction pathways regulating cell cycling processes, cellular migration, and receptor signalling, as well as some very common potential upstream kinases, including several members of the MAP kinase family. The effect of PTK787/ZK 222584 on the kinase activity profile of MSCs was validated by western blotting of key phosphorylated proteins. Moreover, the Pepchip data correspond with the observed inhibitory effects of PTK787/ZK 222584 on proliferation and migration of MSCs. All together, these results confirm the usability of Pepchip array analysis as a powerful tool for kinase activity profiling in functional studies.

We hypothesized that PTK787/ZK 222584 would inhibit outgrowth and function of MSCs by inhibiting PDGFR-signalling. However, decreased peptide-phosphorylation of PDGFR-derived peptides was not observed. Overall, we found very little peptides related to growth factor receptor-kinases (receptor-kinases that can initiate signal transduction after growth factor binding) that showed decreased posphorylation upon addition of PTK787/ZK 222584, while many peptides related to kinases involved in signalling downstream of receptor-kinase showed decreased phosphorylation. These findings may be explained by the assumption that one hour of incubation with PTK787/ZK 222584 may be too long to detect early effects of PTK787/ZK 222584 on the kinase activity of the receptor-related peptides. Conversely, it may be more likely to identify down-modulated kinases in pathways more downstream of the receptors, such as the pathway regulating cell cycling.

We show that the *in vitro* migration capacity of MSCs is impaired by PTK787/ZK 222584. Research in mice has shown that MSCs can be mobilized from the bone marrow into the blood stream (10;11) and that circulating MSCs are able to incorporate into tumor metastases in the lung (5), indicating that MSCs have the capacity to migrate into as well as out of the circulation. We hypothesize that treatment with PTK787/ZK 222584 would inhibit migration of MSCs *in vivo*.

We evaluated the effects of PTK787/ZK 222584 on the outgrowth of hematopoietic precursor cells as a possible negative side-effect of in vivo use. Hematopoietic colony formation decreased, though, remained stable at 50% compared to control at higher concentrations of PTK787/ZK 222584. This result indicates that treatment with PTK787/ZK 222584 may slightly affect but never abolish hematopoiesis in vivo. This assumption is confirmed by the observation that, in animal experiments, PTK787/ZK 222584 is very well tolerated and does not have any significant effects on circulating blood cells or bone marrow leukocytes as a single agent after 21 days of treatment or impair hematopoietic recovery after concomitant cytotoxic anti-cancer agent challenge (21).

Conclusion

We show that the small molecule tyrosine kinase inhibitor PTK787/ZK 222584 may be an important compound to target MSCs for anti-cancer therapies. We show that PTK787/ZK 222584 inhibits outgrowth and function of MSCs, by inhibiting intracellular signalling processes regulating cell cycling and migration. We show the usability of Pepchip array analysis as a powerful tool for kinase activity profiling in functional studies. Besides direct inhibition of tumor cell growth by PTK787/ZK 222584, inhibition of MSCs by PTK787/ZK 222584 and their contribution to the tumor micro-environment and tumor growth may be an additional strategy in the treatment of cancer in the future.

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Chapter 4

Mesenchymal stem cells contribute to tumor cell proliferation by direct cell-cell contact interactions

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Abstract

Bone marrow-derived mesenchymal stem cells (MSCs) have been implicated in tumor progression, making MSCs important targets for anti-cancer strategies. In this study, we show that MSCs promote tumor growth in vivo in a lymphoma xenograft model. We show that MSCs provide direct cell-cell contact interactions and to a lesser extend soluble factors that promote tumor cell proliferation and survival in vitro. PTK787/ZK 222584 reduces tumor growth-promoting effects of MSCs both in vitro and in vivo. Our results address the importance of targeting the MSCs for future anti-cancer strategies.

Introduction

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) are of increasing interest for future therapeutic exploitation in regenerative medicine (1;2), for the treatment of auto-immune diseases and the reduction of graft-versus-host disease in the setting of allogeneic hematopoietic stem cell transplantations (3;4). This interest resides in MSCs' ability to be easily isolated and expanded ex vivo and their multipotential differentiation capabilities together with their immunosuppressive properties (5).

A potential unwanted side-effect of MSCs is, however, their contribution to tumor growth and progression. In mouse models, MSCs have been shown to contribute to growth of chronic lymphoid leukaemia, adenocarcinoma and melanoma cell lines (6-8). Results from Kawada et al. suggest that MSCs can migrate from the bone marrow into the peripheral circulation (9). In mice, intravenously injected MSCs have been detected in the tumor micro-environment of melanoma lung metastases (10;11) and pancreatic carcinoma (12). BM-derived MSCs are thought to be co-recruited towards tumors along with BM-derived hematopoietic and endothelial progenitor cells to establish a micro-environment that supports tumor growth and progression, indicating the importance to target BM-derived MSCs for future anti-cancer strategies.

The mechanism by with MSCs would promote tumor growth is complex. MSCs are thought to facilitate tumor growth by modulating the immune system. MSCs have been shown to suppress the immune system by inhibiting proliferation and activation of T cells and differentiation of dendritic cells and as a consequence tumor cells may escape from recognition by the immune system (5;6). On the other hand, research indicates that MSCs contribute to the tumor micro-environment, for example by promoting tumor vessel formation. MSCs are known to produce pro-angiogenic growth factors (13) and, in mice, MSCs have been associated with increased tumor vessel density (12;14). Alternatively, MSCs are suggested to modulate tumor growth by direct interactions with tumor cells. The mechanism by which MSCs interact with tumor cells, however, remains unknown.

In the present study, we used an *in vitro* transwell co-culturing system to investigate the mechanism by which MSCs directly interact with tumor cells. In addition, we used an *in vivo* co-implantation model to investigate the effect of MSCs on lymphoma

tumor growth (human Daudi Burkitt lymphoma cell line) in immunodeficient NOD/SCID mice. We demonstrate that MSCs provide direct cell-cell-contact interactions by which tumor cell proliferation and survival is promoted. PTK787/ZK 222584 abolished the effect of MSCs on tumor cell survival *in vitro* and reduced MSC-promoted tumor growth *in vivo*. Our results confirm an important role for MSCs in establishing a supportive tumor micro-environment and address the importance of targeting the tumor micro-environment for future anti-cancer strategies.

Materials and Methods

Tumor cell line - Daudi cell line is a well-characterized B lymphoblast cell line (phenotype CD3-, CD14-, CD10+, CD19+, CD20+) derived from a male with Burkitt's lymphoma (kindly provided by Dr. C. Melief, Leiden University Medical Center, Leiden, the Netherlands (15)). The cells were cultured in Iscove's media supplemented with 2 mM L-glutamine, 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM ß-mercaptoethanol.

Generation, characterization and differentiation of MSCs - As described previously (16), bone marrow suspension cells were obtained from sternal marrow aspirations from donors who underwent thorax surgery. Mononuclear cells (MNCs) were isolated and plated in T25 flasks at a density of $1x10^6$/ml and cultured in EBM2 supplemented with EGM2-MV SingleQuots with final serum concentrations of 5% (Cambrex, Clonetics, NY, USA) and 100 U/ml Pencilline and 100 µg/ml Streptomycin (Invitrogen, Gibco, Breda, The Netherlands). Direct immuno-fluorescence using the FACS Calibur (BD Biosciences, Alphen aan de Rijn, The Netherlands) showed that cultured adherent cells were positive for CD90, CD29, CD106, CD140b, CD146, and CD105, and negative for CD45, CD34, CD14 and CD31, which is typical for mesenchymal stem cells (MSCs). Upon stimulation, cultured adherent cells were able to differentiate into adipocytes and osteocytes (16).

In vivo model - Non-obese diabetic–severe combined immunodeficient (NOD/SCID) mice (kindly provided by Dr. L. D. Shultz, The Jackson Laboratory, Bar Harbor, ME)

were bred and maintained in a pathogen-free environment at the Central Animal Facility, University of Groningen. All procedures involving animals were performed in accordance with local ethical animal laws and policies. During the experiment, mice were kept under laminar flow conditions. Eight week old mice were sublethally irradiated (2 Gy) and were subcutaneously injected with tumor cell suspensions at their right flanks. Tumor cell suspensions (100 ul in phosphate-buffered saline (PBS)) consist of 10 x 10^6 Daudi cells admixed with 1 x 10^6 4^{th} passage human MSCs (n=24) or 10 x 10^6 Daudi cells alone (n=24). Eight mice from each group were treated with 25 mg/kg PTK787/ZK 222584 (PTK) through intraperitoneal (i.p.) route every other day, starting on the day of tumor inoculation. PTK was dissolved in DMSO-1% Tween80 (40 mg/ml). Prior to infusion PTK was diluted in PBS. Eight mice from the DM group were injected with GFP-transduced MSCs to track the fate of MSCs *in vivo*. Another four mice were injected with MSCs only as controls for the outgrowth of MSCs. Mice were evaluated for tumor growth every 2 days. Three weeks after tumor inoculation, mice were sacrificed and tumors (when applicable), livers, spleens and bone marrow were removed. Half of each tumor was embedded in TissueTek (Sakura Finetek Europe, Zoeterwoude, The Netherlands), snapfrozen in melting isopentane and stored at -80°C. The other half was fixed in 10% neutral buffered formalin solution for histologic preparations.

Transduction of MSCs with GFP - Third passage MSCs were transduced using FUGENE HD transfection reagent (Roche, Woerden, The Netherlands). MSCs were incubated with retroviral supernatants produced by 293T cells after cofransfection of 2 µg packaging plasmid pCIAmpho and 2 µg reporter plasmid; the murine stem cell virus (MSCV) retroviral expression vector, containing an encephalo-myelocarditis virus (EMCV)–derived internal ribosomal entry site (IRES2) in front of the enhanced green fluorescent protein (EGFP) (pMSCViGFP). Retroviral supernatants were passed through a 0.45 µm filter before addition to MSCs. MSCs were incubated with retroviral supernatant supplemented with 8 µg/ml polybrene for 8 hours in two transduction rounds. Transduction efficiency was 40%.

FACS analysis - From tumors with GFP-transduced MSCs a third part was passed over a 70 µm cell strainer (BD Biosciences, Alphen aan de Rijn, The Netherlands)

and diluted in PBS/5%FCS. Cell suspensions were transferred into 4 ml tubes, centrifugated and resuspended in 300 µl PBS/EDTA and analysed by FACS Calibur (BD Biosciences, Alphen aan de Rijn, The Netherlands) using Flowjo software (Tree Star, Inc., USA).

(Q)RT-PCR - RNA was isolated from tumors, using Machery Nagel RNA isolation kit (Machery Nagel, Düren, Germany), according to the protocol provided. RNA was reverse transcribed with Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Roche Diagnostics). cDNA was prepared at 37°C for at least one hour in a 20 µl reaction mixture containing 2 µg of total RNA, random hexamers, (Pfizer, Capelle a/d IJssel, The Netherlands), 5x first strand buffer, RNAsin and reverse transcriptase (Gibco BRL, Grand Island, NY, USA). Two µl aliquots of cDNA were amplified using iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands) on a MyIQ thermocycler (Bio-Rad, Hercules, CA, USA) and quantified using MyIQ software. GFP expression was assessed using the following primer combinations (Invitrogen, Breda, The Netherlands); forward primer TCCAGGAGCGCACCATCTTC and reverse primer ATGCGGTTCACCAGGGTGTC. A standard curve was made using samples with a known proportion of GFP-positive MSCs mixed with GFP-negative Daudi tumor cells.

Immunohistochemical stainings - Fresh serial tumor sections (4-µm) were thaw mounted on uncoated glass slides. Air-dried sections were fixed in acetone, rinsed and stained with different antibodies diluted in PBS/1% BSA. An appropriate biotin-labelled secondary antibody was used, followed by amplification with biotin-streptavidin ABComplex/HRPO (DAKO AS, Glostrup, Denmark) and subsequent addition of 3-amino-9-ethylcarbazole (substrate for peroxidase) (Sigma, Munchen, Germany). The following antibodies were used: mouse antihuman CD20 and CD22 (1:1000); mouse antihuman desmin (1:200) (all from BD Pharmingen, Breda, The Netherlands); rabbit antihuman FVIII (1:3200; DAKO AS, Glostrup, Denmark) and goat polyclonal Ki67 (1:200; Santa Cruz, Heidelberg, Germany). The first three were previously coupled to secondary antibodies by using a MOM-kit (DAKO cytomation ARK animal research kit; DAKO AS, Glostrup, Denmark). Negative controls were incubated with non-specific IgG as the primary antibody.

Vessel density - Vessel number was assessed using light microscopy in areas of the slide containing the highest numbers of small blood vessels (including sinusoids and capillaries), as previously described (17). Briefly, after identification of the areas with the highest number of capillaries representing the most intense microvasculature (hotspots), the total vessel count was evaluated in these areas over five high-power fields (HPF; 400x) by two double-blind investigators. The variability between the investigators for the vessel count was rho = 0.93. The mean of the two independent counts was considered to be the final measurement for each counting field and hot spot.

Proteome profiler array - Proteome profiler arrays can be used for parallel determination of the relative phosphorylation levels of multiple kinases on one array. Human specific phospho-kinase levels in Daudi tumor with MSCs (DM) and Daudi control (DC) tumors were determined using a human phospho-kinase array kit (R&D Systems, Abingdon, UK) according to the protocols provided. Two DM-tumor lysates were compared with 2 DC-tumor lysates. Spot densities were quantified with Scanalyze software and exported to Microsoft Excel. Spot densities were corrected for the individual background to diminish interarray variances.

Proliferation and apoptosis assays - Daudi tumor cells were plated in 24-well plates (300.000/well) in serum-free Iscove's media supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM ß-mercaptoethanol. Daudi tumor cells were plated a) alone, b) in co-culture on top of a monolayer of MSCs (50.000/well plated the day before) or c) in a transwell system with a 0.4 µm pore size transwell membrane (Costar, Corning Inc. NY, USA) with 50.000 MSCs plated on top of the membrane the day before. To investigate the effect of PTK787/ZK 222584 (PTK) (18) on tumor cell proliferation *in vitro*, 1 or 10 µM PTK was added to the culture medium. PTK was prediluted in DMSO in such way that the volume ratio of DMSO:medium added to the cultures did not exceed 1:1000. In control cultures, an equal volume of DMSO was added as a control for DMSO-induced cell death. MSCs from 6 different donors were used in two independent experiments. After 24 hours Daudi tumor cells were harvested, transferred into 4 ml tubes, centrifugated, resuspended in 200 µl Annexin-V-FLUOS labelling solution (Annexin-V-FLUOS

staining kit, Roche, Woerden, The Netherlands) and incubated for 15 minutes at RT. Cell suspensions were co-stained with CD45-PerCP (BD Pharmingen, Alphen aan de Rijn, The Netherlands) for 15 minutes at RT to separate CD45+ Daudi tumor cells from potentially contaminating CD45-negative MSCs. Reference beads were added and both cells and beads were counted on the FACS Calibur (BD Biosciences, Alphen aan de Rijn, The Netherlands). Data were analysed by the Winlist software (Verity Software).

Survival assay - The direct effect of PTK787/ZK 222584 (PTK) on Daudi tumor cell survival was assessed using a WST assay according to the protocol provided (Roche, Woerden, The Netherlands). Daudi cells were subjected to 0, 1, 5, 10 and 50 µM PTK for 24 hours (6 replicates for each concentration). After addition of the WST-1 cell survival reagent the absorbance was measured at 440 nm in a microplate reader (Microplate Reader Benchmark, Bio-Rad, USA).

Statistical analysis - X-square test was used to analyse differences in tumor incidences. Mann-Whitney-U test was used to analyse differences in tumor weight, vessel density and *in vitro* experiments. P-values < 0.05 were considered statistically significant.

Results

Co-injection of MSCs and tumor cells promote tumor engraftment and growth.

To study the effect of MSCs on tumor growth, Daudi tumor cells (D) or a mixture of MSCs and Daudi tumor cells (DM) were s.c. injected in NOD/SCID mice. Co-injection of MSCs and tumor cells resulted in increased tumor incidence: on day 8, 11 and 13 significantly more mice co-injected with Daudi tumor cells and MSCs (DM-group) had developed tumors compared to mice injected with tumor cells alone (D-group) ($p<0.05$) (figure 1A). At the endpoint of the study, 16/16 DM-mice and 12/16 D-mice had developed tumors that could be taken out for further analysis. The presence of MSCs in the tumor cell mixture resulted in significantly increased tumor weights (median tumor weight 620[400-750] mg in DM-group and 430[260-690] mg in D-group, $p=0.011$, figure 1B). Immunohistochemical staining of tumor sections showed expression of CD20/CD22, typical for Daudi tumor cells. No tumor growth was observed in mice injected with MSCs only.

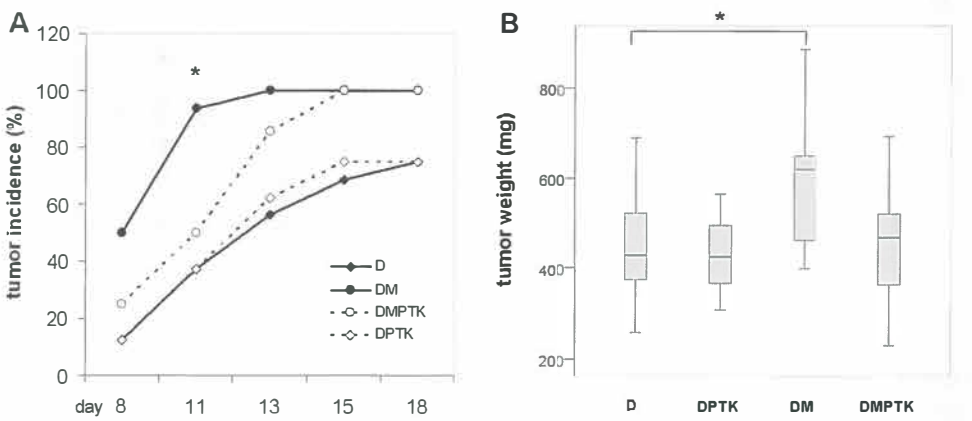

Figure 1: The effect of MSCs on tumor growth. *A) Tumor incidence. Curves represent the percentage of mice that developed visible and palpable tumors. Mice were inoculated with Daudi tumor cells alone (D, n=16) or with Daudi tumor cells and MSCs (DM, n=16), and treated with the small molecule inhibitor PTK787/ZK 222584 (DPTK, n=8 and DMPTK, n=8). *p<0.05 DM vs. other groups. B) Median tumor weight (mg) of indicated groups at the endpoint of the study (day 21). *p<0.011.*

To determine whether MSCs were still present in the tumor at the endpoint of the study, FACS analysis and RT-PCR was performed on tumors from mouse injected with a mixture of Daudi tumor cells and GFP-transduced MSCs. GFP-positive cells were detected by FACS in all tumor cell suspensions of DM-tumors co-injected with GFP-transduced MSCs (5-25 in 1 x $10^{\wedge 6}$ events) (figure 2A). GFP-expression was not detected in cell suspensions from liver, spleen, and bone marrow from these mice. The presence of MSCs in tumor cell suspension was confirmed by quantitative RT-PCR and the proportion of MSCs within the tumor mass was calculated to be about 0.001% - 0.1% (figure 2B and C).

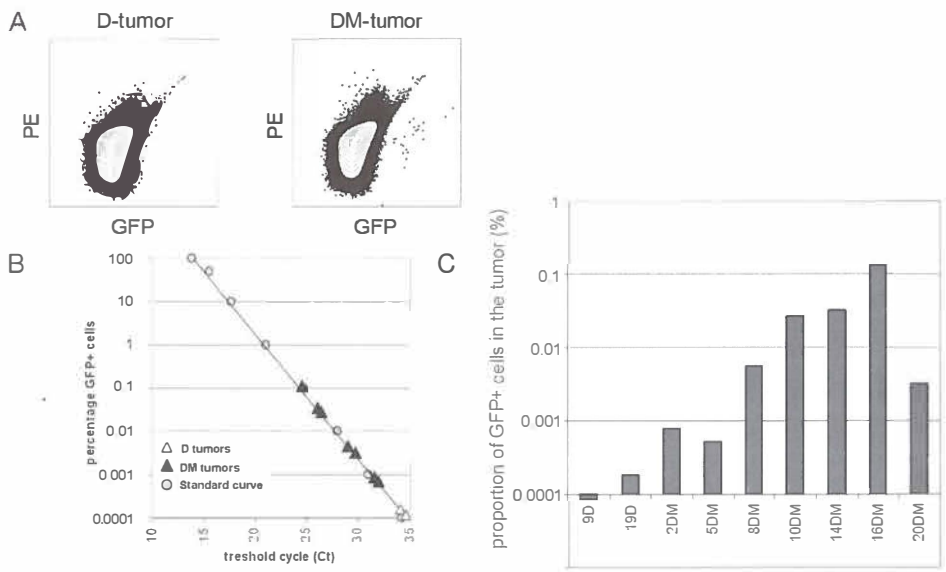

Figure 2: Detection of GFP-transduced MSCs in DM-tumors. *A) FACS-analysis demonstrated the presence of MSCs expressing fluorescent GFP-protein in cell suspensions from DM-tumors (right), but not in cell suspensions from D-tumors (left). B) Quantification of GFP-positive MSCs within the tumor mass by real-time quantitative RT-PCR using SYBR Green dye. A standard curve for the proportion of GFP-positive cells was made using samples with a known proportion of GFP-positive MSCs mixed with GFP-negative Daudi tumor cells. C) The proportion of MSCs within the tumor mass of DM-tumors was calculated to be about 0.001% - 0.1%.*

Increased tumor vessel density in tumors co-injected with MSCs.

It is thought that MSCs can promote tumor vessel formation, thereby indirectly promoting tumor growth. Indeed, we found that the presence of MSCs in the tumor

was associated with significantly increased vessel densities compared to tumors without MSCs as assessed by FVIII-staining (median vessel density 45[3.2-192.3]/HPF in DM-group and 23.8[6.7-68.2]/HPF in D-group, p=0.011) (figure 3). Staining of pericyte coverage of tumor blood vessels as an indicator of vessel maturation, showed a very low numbers of desmin-positive vessels in both D-tumors and DM-tumors (median 5[2.5-12.5] in DM-group and 3.4[0.8-23.5] in D-group). These data indicate that the presence of MSCs in the tumor microenvironment is associated with increased angiogenic sprouting of relatively immature vessels.

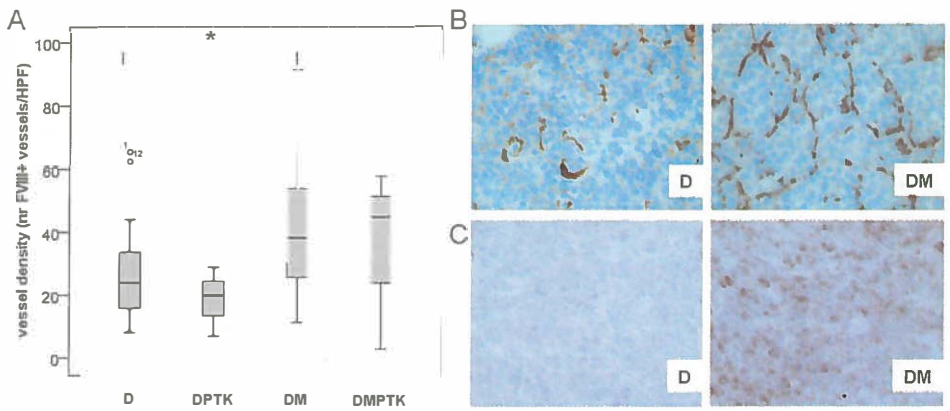

Figure 3: Vessel density. *A) Bars represent median ± range of factor VIII-positive vessels in tumors co-injected with MSCs (DM, n=16) or without MSCs (D, n=12), and in tumors from mice treated with PTK787/ZK 222584 (DPTK, n=6 and DMPTK, n=8). After identification of the areas with the highest number of capillaries in the tumor sections, factor VIII-positive vessels were counted in these areas and evaluated over six high-power fields (400x) per section by two double blind investigators. *p=0.011. B) Representative pictures of factor VIII-stained sections of a D-tumor (left) and a DM-tumor (right). C) Representative pictures of Ki67-stained sections of a D-tumor (left) and a DM-tumor (right).*

Increased proliferation in tumors co-injected with MSCs.

To compare tumor cell proliferation in tumors with and without MSCs, we performed Ki67-staining. Ki67 is a marker strictly associated with proliferation which is present during all active phases of the cell cycle. Ki67-staining on tumor sections showed increased expression of Ki67 in tumors with MSCs compared to control tumors without MSCs (figure 3C). To extent on this result, we performed proteome profiling

analysis on tumors with and without MSCs. These arrays showed increased levels of phosphorylated MEK1/2 (S218/S222, S222/S226), ERK1/2 (T202/Y204, T185/Y187), MSK1/2 (S376,S360), CREB (S133), FAK (Y397) and Src (Y419) in tumors with MSCs compared to tumors without MSCs (figure 4). MEK1/2, ERK1/2 and MSK1/2 are key components of the intracellular ERK-signalling pathway that is crucial for cell proliferation and survival. In addition, levels of phosphorylated $p27^{KIP1}$ (T198 and T157) were decreased in tumors with MSCs compared to tumors without MSCs. Interestingly, induction of $p27^{KIP1}$ (19) has been associated with cell cycle arrest, cell differentiation, apoptosis, and senescence. Together, these results thus strongly suggest that the presence of MSCs in the tumor microenvironment modulates intracellular signalling pathways in the tumor cells that promote tumor cell proliferation and survival.

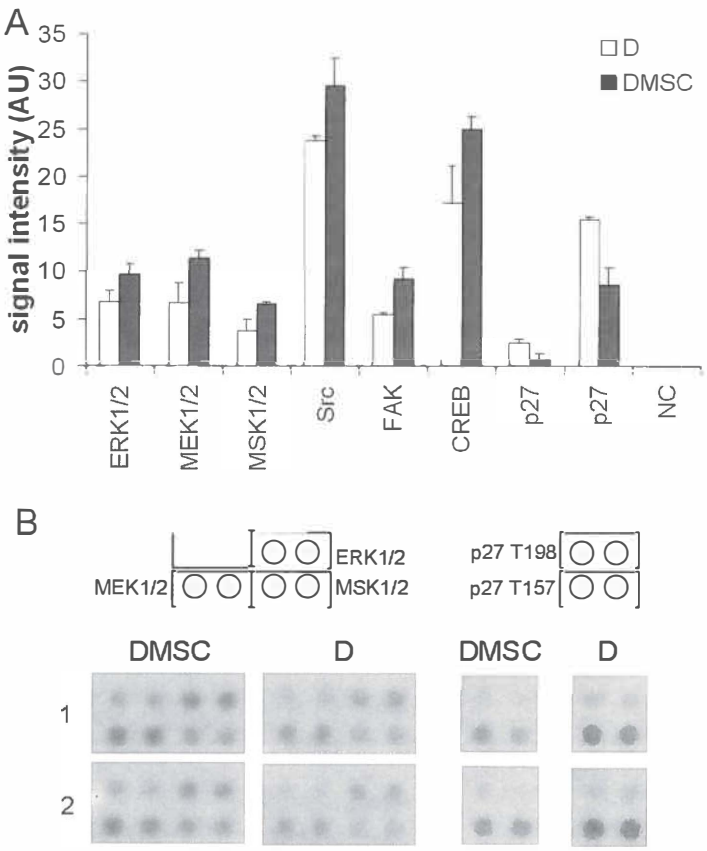

Figure 4: Human phospho-kinase array. *A) Levels of phosphorylated kinases in tumors with MSCs (DMSC) and control tumors without MSCs (D) as determined by a proteome profiler array for human specific intracellular phospho-kinases. Bars represent the mean signal intensity (arbitrary units: AU) ± stdev. B) Examples of array spots of indicated kinases All spots are present on the array in duplicate. Two tumor samples per group were analysed.*

MSCs promote tumor cell growth and protect against tumor cell apoptosis.

To investigate the direct effects of MSCs on tumor cell growth, we compared Daudi tumor cell growth and survival cultured without MSCs (control), in co-culture on top of an adherent layer of MSCs (co-culture) or in co-culture physically separated from MSCs using a transwell culturing system (transwell) for 24 hours in serum-free conditions. In the transwell, the absolute tumor cell number was increased by 12.9 ± 11.2% compared to cultures without MSCs (figure 5B), while in co-cultures, tumor cell number was significantly increased by 20.4 ± 11.5% compared to cultures without MSCs (p=0.01). These data indicate that soluble factors produced by MSCs play a role in promoting Daudi tumor cell proliferation *in vitro*, but that direct cell contact is necessary for the maximal effect.

Culturing tumor cells in serum-free conditions induced tumor cell apoptosis in all culture-types (figure 5C). In transwell cultures with tumor cells cultured separately from MSCs, the percentage of Annexin-V-positive early apoptotic cells was significantly reduced compared to cultures without MSCs (6.54 ± 0.72% and 8.84 ± 0.68% respectively, p=0.02). In co-cultures with tumor cells cultured on top of MSCs allowing direct cell contact, the percentage of early apoptotic tumor cells was even more reduced (3.40 ± 0.28%, p=0.004). In summary, our data demonstrate that Daudi tumor cells are protected against apoptosis when cultured with MSCs. In addition, we show that this effect is partly mediated by MSC-derived soluble factors, but that the maximal protective effect of MSCs on Daudi tumor cell apoptosis is achieved by direct cell contact.

PTK787/ZK 222584 abolishes the protective effect of MSCs on tumor cell apoptosis. In a previous study, we showed that the small molecule tyrosine kinase inhibitor PTK787/ZK222584 (PTK) inhibits proliferation and function of MSCs, and induces apoptosis of MSCs *in vitro* (16). Using a WST cell survival assay, we assessed the direct effect of PTK on Daudi tumor cell survival *in vitro*. Figure 5A

shows that PTK-concentrations up to 5 µM do not significantly induce Daudi tumor cell death. To investigate the effect of PTK on proliferation and apoptosis of Daudi tumor cells cultured with and without MSCs, we added PTK to Daudi tumor cells cultured without MSCs (control), in co-culture on top of an adherent layer of MSCs (co-culture) or in co-culture physically separated from MSCs using a transwell culturing system (transwell) for 24 hours in serum-free conditions.

Administration of 1 µM PTK to cultures without MSCs did not induce extra Daudi tumor cell apoptosis compared to DMSO-control (8.76 ± 0.36% and 8.84 ± 0.68% respectively). In the co-cultures, however, addition of 1 µM PTK significantly increased the percentage of early apoptotic tumor cells compared to DMSO-control (7.44 ± 0.30% and 3.40 ± 0.28% respectively, p=0.004). Administration of 10 µM PTK significantly induced tumor cell death in all culture-types compared to DSMO-control cultures (p=0.004) (figure 5C). Interestingly, upon administration of PTK, we observed similar percentages of early apoptotic tumor cells in all culture-types. These results suggest that PTK abolished the protective effects of MSCs against Daudi tumor cell apoptosis.

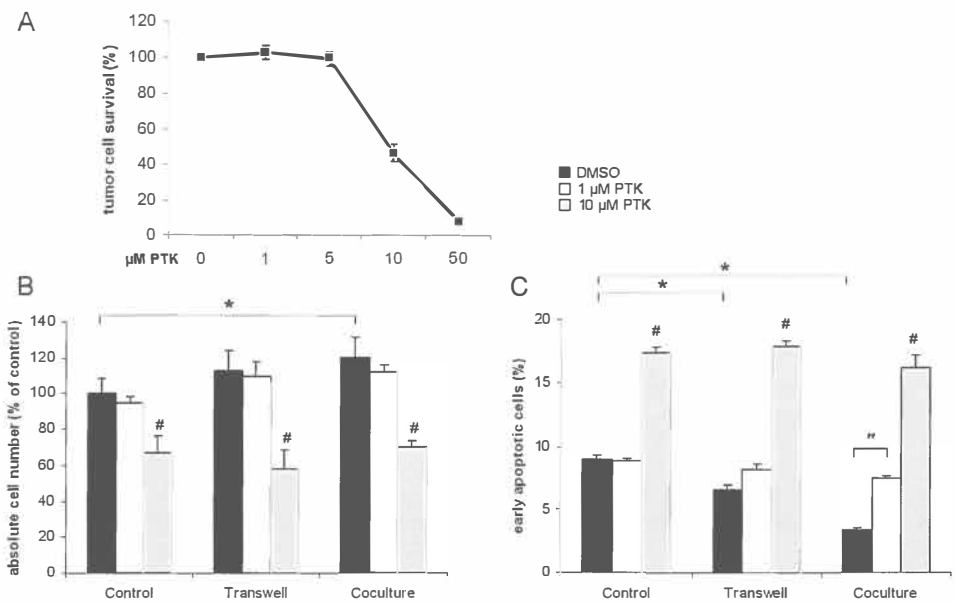

Figure 5: In vitro co-culturing experiments. *A) The effect of PTK787/ZK 222584 on Daudi tumor cell survival as assessed by a WST-assay. Data are normalized to the DMSO-control (100%). B and C) The effect of PTK787/ZK 222584 on Daudi tumor cell proliferation (B) and apoptosis (C). Daudi tumor cells were plated alone*

*(control), in co-culture on top of a monolayer of MSCs (co-culture), or in a transwell system (transwell). After 24 hours tumor cells were harvested and stained with Annexin-V/ PI and co-stained with CD45-PerCP to separate CD45+ tumor cells from potentially contaminated CD45- MSCs. Reference beads were added for absolute cell counts. In graph B, the Y-axis represents the absolute tumor cell number ± stdev in indicated culture types. Data are normalized to the culture without MSCs and without PTK787/ZK 222584 (100%). In graph C, the Y-axis represents the percentage of Annexin-V-positive early apoptotic tumor cells ± stdev in indicated culture types. Black bars represent cultures without PTK787/ZK 222584; white bars represent cultures with 1 μM PTK; gray bars represent cultures with 10 μM PTK. *p<0.02; #p=0.004 10 μM PTK vs. DMSO-control; "p=0.004.*

PTK787/ZK222584 reduces the effect of MSCs on in vivo tumor growth.

In our *in vivo* model, PTK-treatment of mice injected with Daudi tumor cells alone did not result in significant differences in tumor incidence or tumor weight compared to untreated control mice (figure 1A and B), indicating that, at the dosage used, PTK does not directly inhibit Daudi tumor growth *in vivo*. In DM-mice, however, we observed that treatment with PTK reduced the effect of MSCs on tumor incidence: only 4/8 DM-mice treated with PTK had developed tumors whereas 8/8 untreated control DM-mice had developed tumors at day 11 of tumor inoculation (p=0.046) (figure 1A). In addition, tumor weight tended to be reduced in DM-mice treated with PTK compared to untreated control DM-mice (p=0.08) (figure 1B), indicating that treatment with PTK reduces the effect of MSCs on tumor growth. Vessel density per high power field of D and DM-tumors was similar in PTK-treated and untreated mice.

Discussion

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) are of potential interest for many clinical applications (1-4). A potential unwanted side-effect of MSCs is their contribution to tumor growth and progression.(6-8) We show that MSCs promote tumor growth in a lymphoma xenograft NOD/SCID mouse model. *In vivo* co-implantation models and *in vitro* models combining tumor cells and MSCs hold great promise as a system in which the interaction between tumor and stroma can be manipulated and studied. Immunodeficient mouse models provide the optimal model to study these tumor-stroma interactions without intervention of the immune system. In this study, we focussed on the mechanism of MSC-promoted tumor growth and explored ways to inhibit tumor growth by targeting MSCs. Our results indicate that

MSCs protect tumor cells against apoptosis and promote initial tumor cell proliferation mainly by direct cell-cell contact interactions.

The increased tumor incidence observed in mice injected with a MSC-tumor cell mixture compared to tumor cells alone suggested a very early effect of MSCs on tumor engraftment in our model. To study the early effects of MSCs on Daudi tumor proliferation and survival in more detail we performed *in vitro* co-culture experiments. These experiments showed that MSCs promote proliferation of Daudi tumor cells. Furthermore, we show that apoptosis is reduced when Daudi tumor cells are cultured in contact with MSCs. Similar effects have been observed by Kyriakou et al. showing an increased number of viable Raji (Burkitt lymphoma) tumor cells after 7 days of co-culture with MSCs (20), and Ramasamy et al. showing decreased percentages of apoptotic BV173 (chronic myeloid leukaemia) tumor cells after 24 hours of co-culture with MSCs (8). MSCs are suggested to modulate tumor growth by direct interactions with tumor cells. We hypothesized that MSCs may influence tumor growth by direct cell-contact with tumor cells and/or by providing soluble factors. Using a transwell co-culturing system, we demonstrated that direct cell-contact between MSCs and tumor cells is needed for the maximal effect of MSCs on Daudi tumor cell proliferation and survival, while soluble factors play a relatively minor role.

Ki67-staining showed increased expression of Ki67 in tumors with MSCs compared to control tumors without MSCs, suggesting that MSCs promote tumor growth by promoting tumor cell proliferation. Using a proteome profiler array, we found that phosphorylation levels of several kinases involved in signalling downstream of growth factor receptors (MEK1/2, ERK1/2, MSK1/2 and CREB) were increased in tumors with MSCs compared to tumors without MSCs, indicating activation of growth receptor signalling pathways in tumors with MSCs. In addition, we showed that phosphorylation levels of FAK and Src, which are key kinases involved in integrin receptor signalling were increased in tumors with MSCs compared to tumors without MSCs. MSCs are known to provide a wide variety of growth and survival factors, including epidermal growth factor (EGF), hepatocyte growth factor (HGF) and interleukin-6 (IL-6) that can promote tumor growth (21). Daudi tumor cells express EGF-receptor (data not shown) indicating that EGF produced by MSCs is an important soluble factor mediating the observed effects on Daudi tumor cell proliferation and apoptosis. In addition, MSCs express various integrins and produce

extracellular matrix molecules (ECM). It has been observed that binding of tumor cells to integrins and ECM promotes tumor cell survival (22) and protects them from drug-induced apoptosis (23). Overall, our results indicate that the presence of MSCs in the tumor micro-environment *in vivo* modulates intracellular signalling pathways in the tumor cells that promote tumor cell proliferation and survival.

At the endpoint of the *in vivo* study, MSCs were still present in the tumor as detected by flow cytometry. Using QRT-PCR, the presence of MSCs in the tumor was quantified ranging between 0.001% and 0.1% of the tumor mass. This percentage may under-represent real numbers of MSCs present in the tumor micro-environment because of down-regulation of GFP-expression. Interestingly, we demonstrated that the presence of MSCs in the tumor micro-environment was associated with significantly increased tumor weight and tumor vessel density. Besides direct effects of MSCs on tumor cells, MSCs are thought to promote tumor growth by contributing to tumor vessel formation. MSCs are known to produce pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), which activates and promotes proliferation of endothelial cells (13). In addition, some studies have described trans-differentiation of MSCs into endothelial-like cells (14), pericyte-like cells (24), and tumor-associated fibroblasts (21). In mice, MSCs have been found to contribute to the formation of blood vessels as a response to wounding (25). Similar to wound healing processes, tumor growth also depends on the formation of new blood vessels. Our data confirm earlier data showing that MSCs are associated with increased tumor vessel density (12;14).

Evidence has been accumulating that MSCs and MSC-derived tumor stromal cells, such as endothelial-like cells, pericyte-like cells and tumor-associated fibroblasts are implicated in important aspects of tumor biology, such as neoplastic progression, tumor growth, angiogenesis, and metastasis (reviewed by (26) and (27)), making these cells important targets for anti-cancer strategies. Previously, we showed that growth and function of MSCs is inhibited and apoptosis of MSCs is induced by PTK787/ZK 222584 (16). In the present study, we show that MSCs protect Daudi tumor cells against apoptosis, and that PTK787/ZK 222584 abolishes this effect. Interestingly, *in vivo*, we observed that treatment with PTK787/ZK 222584 resulted in later outgrowth of the tumor and reduced tumor weight in mice injected with a MSC-tumor cell mixture, while PTK787/ZK 222584 did not influence tumor kinetics in the

control-group of mice injected with Daudi tumor cells alone. These data suggest that PTK787/ZK 222584 reduces the effect of MSCs on tumor growth *in vivo* by direct inhibition of MSCs. Although PTK787/ZK 222584 has been extensively studied to target endothelial cells, we did not observe a reduction in tumor vessel density of D-tumors in response to PTK787/ZK 222584-treatment. This may, however, be explained by the low overall vessel density that is characteristic for the Daudi tumor model used. Because of this, we may not be able to observe reductions in tumor vessel density induced by PTK787/ZK 222584 at the dosage used.

Conclusion

In conclusion, we show that direct cell-cell contact interactions between MSCs and tumor cells are important in promoting tumor cell proliferation and survival. Based on our results, we hypothesize that MSCs modulate the tumor micro-environment *in vivo* in such way that tumor cells more easily survive and the outgrowth of the tumor is facilitated. In addition, we show that PTK787/ZK 222584 reduces the effects of MSCs on tumor growth, indicating the importance of targeting the tumor micro-environment, including MSCs, for future anti-cancer strategies.

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Chapter 5

Lack of *in vivo* homing of bone marrow-derived mesenchymal stem cells to subcutaneous lymphoma tumors: VEGF-A does not function as a chemoattractant

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Abstract

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) have been shown to promote tumor growth in various *in vivo* models. *In vitro* research indicated that vascular endothelial growth factor A (VEGF-A), which is highly expressed in many tumors, may be a crucial chemoattractant for BM-derived MSCs. VEGF-A expressing tumors may therefore be of increased risk of infiltration of tumor growth-promoting MSCs compared to tumors with low expression of VEGF-A. In this study, we assessed whether VEGF-A functions as a chemoattractant for circulating human BM-derived MSCs *in vivo*. We investigated direct homing of intravenously (i.v.) injected GFP+ MSCs to subcutaneous (s.c.) VEGF-A overexpressing tumors and control tumors by determining the presence of MSCs in the tumors within 24 hours after injection of the MSCs. We injected MSCs i.v.to mimic the physiological route of bone marrow-derived cells that may migrate towards tumor sites. *In vivo* tumor homing of i.v. injected MSCs was detected neither in VEGF-A-tumors nor in control tumors. Instead, MSCs were massively present in the lungs of 6/6 mice, and small numbers were detected in the spleen of 5/6 mice. Although *in vitro* migration assays showed that migration of MSCs was significantly enhanced by VEGF-A (conditioned medium from VEGF-A-overexpressing Daudi tumor cells increased migration of MSCs by 34.4 ± 15.4% compared to medium from control tumor cells), *in vivo* VEGF-A overexpression by the tumor did not result in increased homing of MSCs compared to control tumors. In conclusion, our data indicate that intravenously injected MSCs do not directly home to subcutaneous tumors in our *in vivo* model, and that VEGF-A overexpression in the tumor does not enhance homing of circulating human BM-derived MSCs.

Introduction

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) are of great clinical interest. MSCs are being evaluated for their application in regenerative medicine (1;2), auto-immune diseases and hematopoietic stem cell (HSC) transplantations (3;4). Although the use of MSCs seems very promising, evidence has been accumulating that this is not without any risk (5). Co-injection of BM-derived MSCs with HSC transplantation is associated with a higher recurrence rate in hematologic malignancy patients (6). In addition, BM-derived MSCs inoculated together with tumor cells have been shown to contribute to the growth of chronic lymphoid leukaemia, adenocarcinoma, melanoma and lymphoma cell lines in murine models (7-10). Besides promoting tumor vessel formation, we previously showed that direct cell-cell contact interactions between MSCs and tumor cells are important in promoting tumor cell proliferation and survival (10).

BM-derived MSCs can be mobilized into the peripheral circulation under certain conditions, such as hypoxia (11). In humans, MSCs have been detected in peripheral blood from breast cancer patients after growth factor mobilization (12;13), as well as in non-mobilized blood from healthy subjects (14). Circulating BM-derived MSCs are proposed to home to and engraft in tumor microenvironments. For example, MSCs have been shown to posses an innate tropism to orthotopic glioma tumors (15) after regional administration of MSCs directly into the carotid artery. Homing to specific sites, such as tumors, is mediated by adhesive interactions as well as chemokines and cytokines released from tumor tissue or endothelial cells (16). In tumors, expression of vascular endothelial growth factor A (VEGF-A) is often increased in response to low oxygen concentrations and subsequent upregulation of the transcription factor HIF 1α (17). *In vitro*, BM-derived MSCs have been shown to migrate in response to a range of soluble factors, including VEGF-A (17-20). Many tumors express VEGF-A, and tumors that highly express VEGF-A may be of increased risk of infiltration of potentially tumor growth promoting BM-derived MSCs compared to tumors with low expression of VEGF-A. To date, the role of VEGF-A in tumor homing of BM-derived MSCs has not been investigated *in vivo.*

In this study, we assessed whether VEGF-A functions as a chemoattractant for circulating human BM-derived MSCs *in vivo*. We investigated direct homing of

intravenously (i.v.) injected GFP+ MSCs to subcutaneous (s.c.) VEGF-A overexpressing tumors and control tumors by determining the presence of MSCs in the tumors within 24 hours after injection of the MSCs. We injected MSCs i.v. to mimic the physiological route of bone marrow-derived cells that may migrate towards tumor sites. Our data indicate that i.v. injected MSCs do not directly home to s.c. tumors in our model, and that tumor VEGF-A does not enhance homing of circulating human BM-derived MSCs to tumors.

Materials and Methods

Generation of the tumor cell lines - Daudi (human Burkitt lymphoma) cells (21) were cultured in Iscove's media supplemented with 2 mM L-glutamine, 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM ß-mercaptoethanol. Stably transduced Daudi-VEGF and Daudi-MOCK cell lines were generated using retroviral supernatants produced by 293T cells after cofransfection of packaging plasmid pClAmpho and reporter plasmid pMSCV-VEGF-A-NGFR and pMSCV-NGFR, respectively. Retroviral supernatants were passed through a 0.45 µm filter before addition to the Daudi cells. Daudi cells were incubated with retroviral supernatant supplemented with 8 µg/ml polybrene for 16 hours in two consecutive transduction rounds. NGFR-positive Daudi cells were sorted using MoFlo XPD Cell Sorter to obtain a purity of 99 ± 1% transduced cells. Quantitative PCR showed that VEGF-A was over-expressed 92.6-fold by Daudi-VEGF-A cells compared to Daudi-MOCK cells (22). The functionality of secreted VEGF-A from transduced cells was assessed by adding its supernatant to HUVEC and to quantify the expression level of the VEGF-A responsive gene EGR3 in HUVEC using real-time PCR as described in detail by Liu et al. (23).

Generation, characterization and transduction of MSCs - As described earlier (24), bone marrow suspension cells were obtained from sternal marrow aspirations from one donor who underwent thorax surgery. Mononuclear cells (MNCs) were isolated and plated in T25 flasks at a density of $1x10^6$/ml and cultured in EBM2 supplemented with EGM2-MV SingleQuots with final serum concentrations of 5% (Cambrex,

Clonetics, NY, USA) and 100 U/ml Pencilline and 100 µg/ml Streptomycin (Invitrogen, Gibco, Breda, The Netherlands). By day 7-8, the culture plate was covered with a confluent layer of adherent cells that were positive for CD90, CD29, CD106, CD140b, CD146, and CD105, and negative for CD45, CD34, CD14 and CD31, which is typical for mesenchymal stem cells (MSCs). Upon stimulation, cultured adherent cells were able to differentiate into adipocytes and osteocytes (24). Third passage MSCs were transduced with retroviral supernatants supplemented with 8 µg/ml polybrene in two transduction rounds of 8 and 12 hours. Retroviral supernatants were produced by 293T cells transfected with 2 µg packaging plasmid pCIAmpho and 2 µg pMSCViGFP using FUGENE HD transfection reagent (Roche, Woerden, The Netherlands). Retroviral supernatants were passed through a 0.45 µm filter before addition to MSCs. Transduction efficiency was 25%.

Isolation and transduction of CD34+ cells - CD34+ cells were isolated from umbilical cord blood MNCs using magnetic bead column separation according to the Mini MACS protocol supplied (Miltenyi Biotec, Bergisch Gladbach, Germany). All cord blood samples were obtained with written, informed consent. Freshly isolated CD34+ cells were prestimulated in HPGM supplemented with c-Kit ligand (K), Flt-3 ligand (F) and thrombopoietin (T) (100 ng/ml each) for 24 hours at 37ºC and 5% CO_2. Prestimulated CD34+ cells were transduced with lentiviral supernatant supplemented with KFT (100 ng.ml each) and 2 µg/ml polybrene in 2 consecutive rounds of 24 hours. Lentiviral supernatants were produced by 293T cells transfected with 3 µg pCMV Δ8.91, 0.7 µg VSV-G and 3 µg pTRIP Renilla RNAi (kind gift from Prof. Dr. H. Spits, Amsterdam, The Netherlands) using FUGENE HD transfection reagent (Roche, Woerden, The Netherlands). Lentiviral supernatants were passed through a 0.45 µm filter before addition to CD34+ cells. Transduction efficiency was 30%.

Migration assay - Transwells were prepared by adding 30 µl Biocoat Matrigel (BD Biosciences, Alphen aan de Rijn, The Netherlands) onto the membrane of 24 wells transmigration chambers (Costar, Corning Inc, NY, USA) and incubating the transwells overnight at room temperature. The lower wells were filled with 500 µl conditioned or control medium with 2% FCS, supplemented with 150 ng/ml recombinant human VEGF-A (Sigma Aldrich, Zwijndrecht, The Netherlands) and/or

15 µg/ml purified goat IgG neutralizing anti-VEGF-A antibodies (R&D Systems, Minneapolis, USA). Matrigel coated membranes were then overlaid with 5 x 10^4 MSCs suspended in 50 µl EBM2 (Cambrex, Clonetics, NY, USA) with 1% FCS. Transwells were incubated overnight in a 37ºC incubator with 5% CO_2. The next day, after removal of the Matrigel, membranes were fixed in methanol for 5 minutes, stained with Giemsa solution (Sigma Aldrich, Zwijndrecht, The Netherlands) for 5 minutes and mounted on a slide. Migrated cells were counted in 6 high power fields (400x) using a microscope with a digital camera Olympus BX50. All experiments were performed in triplicate.

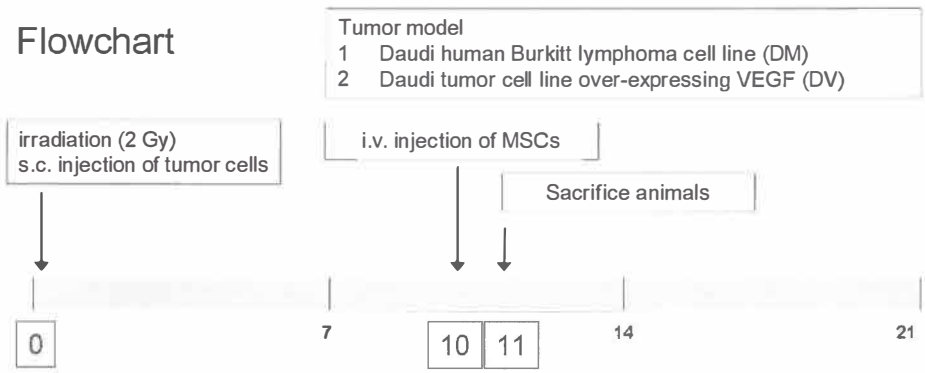

Figure 1: Experimental set up of the in vivo study: *Eight week old mice were sublethally irradiated (2 Gy) and subcutaneously injected with 10 x 10^6 Daudi tumor cell suspensions: Daudi-MOCK (n=6) or Daudi-VEGF (n=6). Ten days after tumor inoculation, half of the mice of each group were intravenously injected with either MSCs ($2*10^6$ in total of which $0.5*10^6$ GFP+). Mice were sacrificed 24 hours later. Tumor, spleen, lungs and bone marrow cells were analyzed for the presence of GFP+ cells.*

In vivo model - Thirty six non-obese diabetic–severe combined immunodeficient (NOD/SCID) mice (Charles River, Maastricht, The Netherlands) were maintained in a pathogen-free environment at the Central Animal Facility, University of Groningen. All procedures involving animals were performed in accordance with local ethical animal laws and policies. During the experiment, mice were kept under laminar flow conditions. Eight week old mice were sublethally irradiated (2 Gy) and were subcutaneously injected with 10 x 10^6 Daudi tumor cell suspensions (100 µl in phosphate-buffered saline (PBS)) at their right flanks. Mice were injected with Daudi-

MOCK (n=6) or Daudi-VEGF-A (n=6). Mice were evaluated for tumor growth every 2 days. Ten days after tumor inoculation, half of the mice of each group were intravenously injected with either MSCs ($2*10^6$ in total of which $0.5*10^6$ GFP+) or CD34+ cells ($0.9*10^6$ in total of which $0.3*10^6$ GFP+). Mice were sacrificed 24 hours later (figure 1). Tumor, spleen and lungs were removed and bone marrow cells were harvested. One half of the tissues was frozen in melting isopentane and stored at -80°C till later use, while the other half was directly processed for FACS analysis.

FACS analysis - Tissues were passed over a 70 µm cell strainer (BD Biosciences, Alphen aan de Rijn, The Netherlands) and diluted in PBS/ 5%FCS to obtain single cell suspensions. Cell suspensions were transferred into 4 ml tubes, centrifugated and resuspended in 300 µl PBS/EDTA. Samples were kept on ice during the whole procedure. Samples were analyzed using FACS LSR-II machine (BD Biosciences, Alphen aan de Rijn, The Netherlands) and Flowjo software (Tree Star, Inc., USA). Two million events were counted for each tissue to detect GFP+ cells at high sensitivity (<10 out of $10^{\wedge 6}$).

(Q)RT-PCR - RNA was isolated from tissues, using Machery Nagel RNA isolation kit (Machery Nagel, Düren, Germany), according to the protocol provided. RNA was reverse transcribed with Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Roche Diagnostics). cDNA was prepared at 37°C for at least one hour in a 20 µl reaction mixture containing 0,75 µg of total RNA, random hexamers, (Pfizer, Capelle a/d IJssel, The Netherlands), 5x first strand buffer, RNAsin and reverse transcriptase (Gibco BRL, Grand Island, NY, USA). cDNA was amplified using iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands) on a MylQ thermocycler (Bio-Rad, Hercules, CA, USA) and quantified using MylQ software. GFP and human specific β-actin expression was assessed using the following primer combinations (Invitrogen, Breda, The Netherlands); GFP forward primer TCCAGGAGCGCACCATCTTC and reverse primer ATGCGGTTCACCAGGGTGTC; human β-actin forward primer GCTGTGCTACGTCGCCCTG and reverse primer GGAGGAGCTGGAAGCAGCC. GFP expression in each lung and bone marrow sample was normalized to mouse specific β-actin expression, and GFP expression in each tumor sample was normalized to human RPL22 expression. Lung, bone

marrow and tumor tissues of mice not injected with GFP+ cells were analysed to determine background levels.

Immunohistochemical stainings - Fresh serial tumor sections (4-µm) were thaw mounted on uncoated glass slides. Air-dried sections were fixed in acetone for 10 min, rinsed and stained with rabbit GFP-antibody (Invitrogen, Gibco, Breda, The Netherlands) diluted (1:200) in PBS/1% BSA. A swine anti-rabbit biotin-labelled secondary antibody (1:100) was used, followed by amplification with biotin-streptavidin ABComplex/HRPO (DAKO AS, Glostrup, Denmark) and subsequent addition of 3-amino-9-ethylcarbazole (substrate for peroxidase) (Sigma, Munchen, Germany). Negative controls were produced by omitting the primary antibody.

Results

Intravenously injected MSCs do not home to VEGF-A overexpressing tumors.

To prove that tumor VEGF-A indeed functions as a chemo-attractant for MSCs, we performed *in vitro* migration assays. Conditioned medium derived from VEGF-A overexpressing (DV) Daudi tumor cells significantly increased migration of MSCs by 34.4 ± 15.4% compared to control Daudi-Mock (DM)-conditioned medium (number of migrated cells: 25.2 ± 2.69 cells/HPF and 19.2 ± 0.69 cells/HPF, respectively) (p=0.01) (figure 2B). These results indicate that migration of MSCs is increased in response to tumor VEGF-A. Inhibition of VEGF-A by anti-VEGF-A antibodies reduced this effect (p=0.036).

To assess whether VEGF-A functions as a chemoattractant for circulating human BM-derived MSCs *in vivo,* we investigated direct homing of intravenously (i.v.) injected GFP+ MSCs to subcutaneous VEGF-A overexpressing tumors (DV-tumors) and control tumors (DM-tumors). Direct tumor homing within 24 hours after injection of the MSCs was detected neither in DV-tumors nor in DM-tumors. No GFP+ cells were detected by FACS in two out of three DM-tumors (table 1). Five GFP+ events were counted in one DM-tumor. The GFP expression level, however, was low and close to the border of the gate (figure 2A). Although *in vitro* migration of MSCs to VEGF-A was significant, *in vivo* VEGF-A overexpression by the tumor did not result in increased tumor homing of MSCs compared to control tumors (figure 2A and table 1). No GFP+ cells were detected by FACS in two out of three DV-tumors (table 1) and only 2 GFP+ events were counted in one DV-tumor (figure 2A). To confirm the data obtained by FACS analysis, QRT-PCR was performed to detect GFP-reporter RNA in the tumors. GFP expression could not be detected in any tumor (figure 2C). In addition, GFP+ cells were also not detected in any tumor after immunostaining of GFP (figure 2D). These data indicate that, in our model, intravenously injected MSCs do not directly home to subcutaneous tumors and that VEGF-A overexpression by the tumor does not enhance homing of MSCs to the tumor.

Intravenously injected MSCs massively localize to the lungs.

FACS analysis showed high numbers of GFP+ MSCs in the lungs of all mice 24 hours after injection of the MSCs [range 2437-5482 cells per 10^6 events] (figure 3A

and table 1). Immunostaining of GFP on lung sections showed massive infiltration of GFP+ MSCs in the lungs of MSCs-injected mice (figure 3C). GFP+ MSCs were observed throughout the whole lung, whereas they were not detected in lungs from non-injected control mice. QRT-PCR confirmed the presence of GFP+ MSCs in the lungs of all mice. Obvious expression of GFP as well as human β-actin (used as another method to measure the presence of MSCs in Daudi tumors) was detected in the lungs of all mice (figure 3B). Human β-actin expression was not detected in lungs of tumor bearing control mice that were not injected with MSCs, indicating that the detected expression of human β-actin is not derived from migrated human Daudi tumor cells.

Intravenously injected MSCs sporadically home to the spleen.

FACS analysis showed the presence of GFP+ MSCs in the spleen in five out of six mice, as determined 24 hours after injection of the MSCs [range 6-51 cells per 10^6 events] (table 1, figure 4). This result demonstrates that not all intravenously injected MSCs accumulate in the lungs without any cell passing through. It also suggests that MSCs may even prefer homing to the spleen instead of the tumor. In the bone marrow, less than 10 GFP+ events were counted by FACS in four out of six mice (table 1, figure 4), indicating that intravenously injected MSCs can reach the bone marrow microenvironment but do not specifically home to the bone marrow.

MSC	24h	POS EVENTS per 10^6		
mouse	tumor	spleen	lungs	bone marrow
DM	5	0	2437	5
DM	0	43	2997	0
DM	0	21	5482	2
DV	0	51	3048	6
DV	2	6	3062	0
DV	0	34	2453	3

Table 1: Detection of GFP+ MSCs by FACS analysis. *Absolute number of GFP+ MSCs per million events in single cells suspensions from indicated tissues 24 hours after intravenous injection of GFP+ MSCs in mice. Data are corrected for background using tissues from control mice that were not injected with MSCs or CD34+ cells. Mice were inoculated with Daudi-Mock tumors (DM) or Daudi-VEGF tumors (DV).*

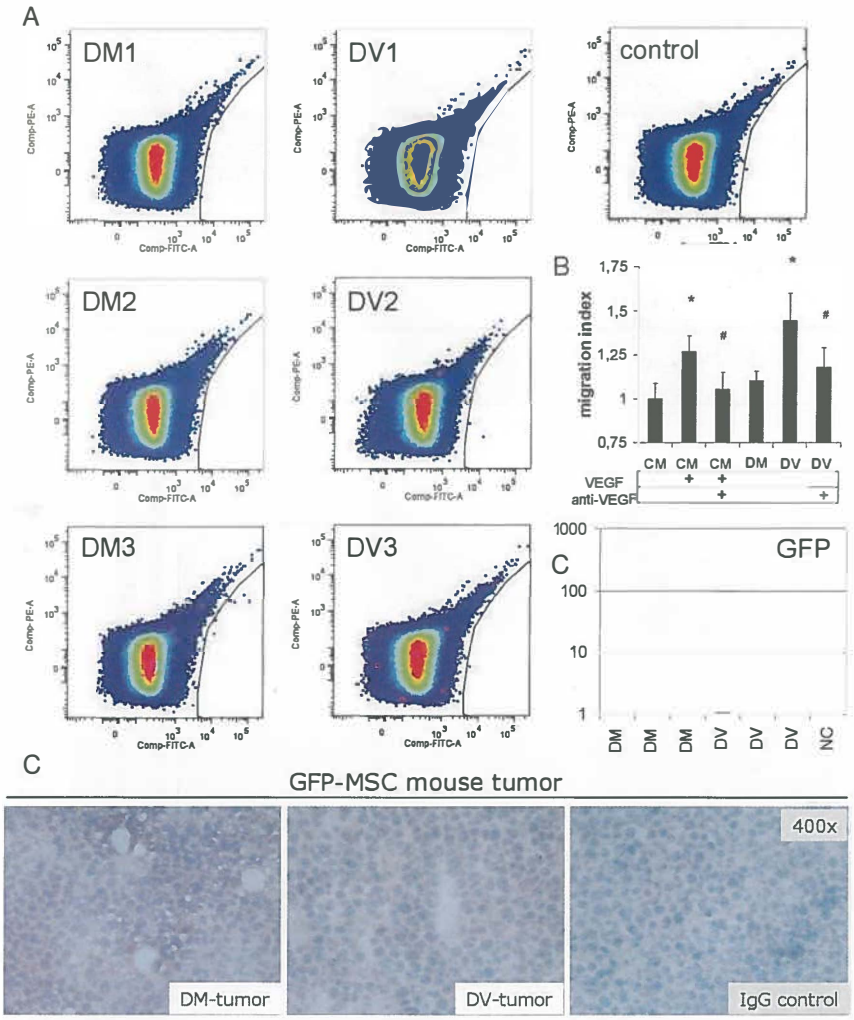

Figure 2: Detection of GFP+ MSCs in tumors. *A) FACS-plots of single cell suspensions from tumor tissues 24 hours after intravenous injection of GFP-labelled MSCs in mice. Mice that were not injected with MSCs served as control (control). B) Transwell migration assay. MSCs were allowed to migrate for 20 hours towards conditioned medium derived from the Daudi-MOCK tumor cell line (DM), from the Daudi-VEGF tumor cell line (DV), or from control medium (CM). Media were supplemented with 150 ng/ml VEGF-A and/ or with 15 ug/ml anti-VEGF-A antibodies as indicated. The average number of migrated cells was normalized to the number of migrated cells towards Daudi-MOCK control medium (migration index of 1). C) Relative GFP expression in tumors from MSC injected mice as assessed by QRT-PCR. Expression levels were normalized to human RPL22 expression to correct for the RNA content, and compared to background levels (Ct-value: 34.38) using corresponding tissues from control mice that were not injected with MSCs (NC). D) Immunohistochemical staining of tumor sections using GFP-antibody. GFP+ cells could not be detected. IgG-staining was performed as negative control. Mice were inoculated with Daudi-Mock tumors (DM) or Daudi-VEGF tumors (DV). Magnification 400x.*

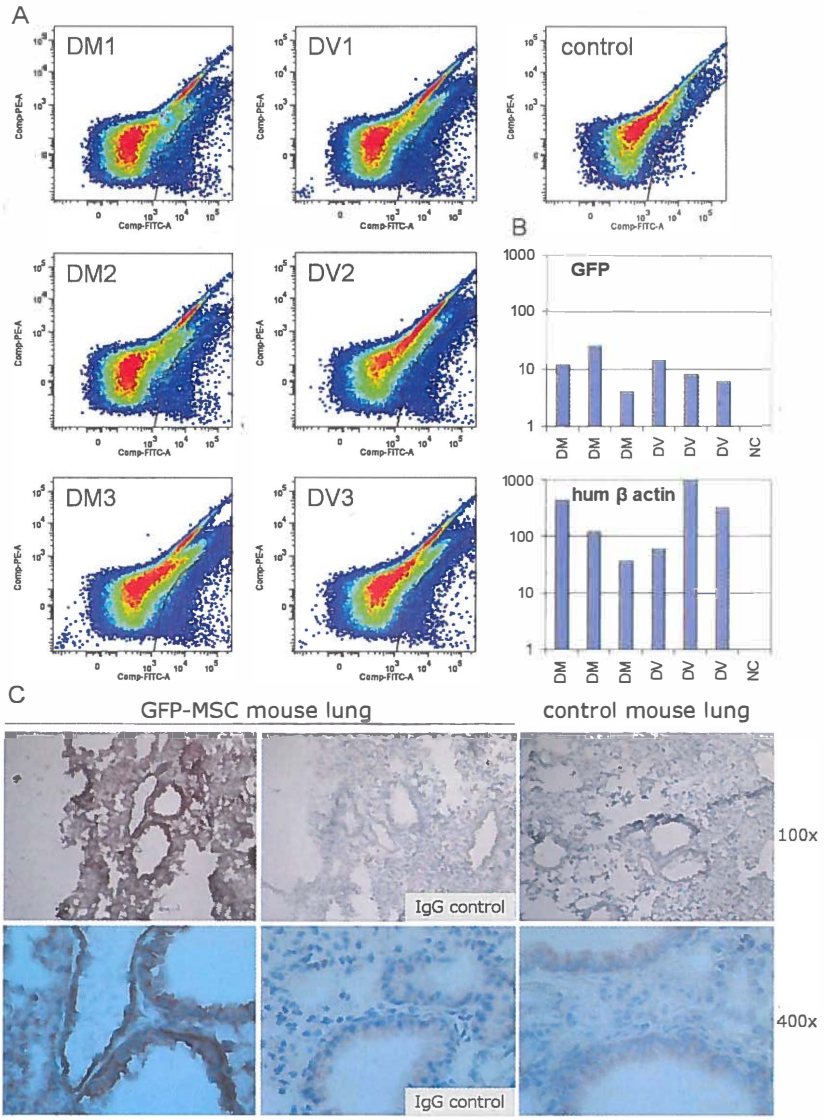

Figure 3: Detection of GFP+ MSCs in lungs. *A) FACS-plots of single cell suspensions from lung tissues 24 hours after intravenous injection of GFP-labelled MSCs in mice. Mice that were not injected with MSCs served as control (control). B) Relative GFP expression and relative human β-actin expression in lungs from MSC injected mice as assessed by QRT-PCR. Expression levels were normalized to mouse β-actin expression to correct for the RNA content, and compared to background levels (Ct-value: GFP 35.16; β-actin 30.12) using corresponding tissues from control mice that were not injected with MSCs (NC). C) Immunohistochemical staining of lung sections using GFP-antibody. GFP+ cells were detected throughout the whole lung. GFP-staining of lungs from control mice (not injected with GFP+ cells) and IgG-stainings were performed as negative controls. Mice were inoculated with Daudi-Mock tumors (DM) or Daudi-VEGF tumors (DV). Magnification 400x.*

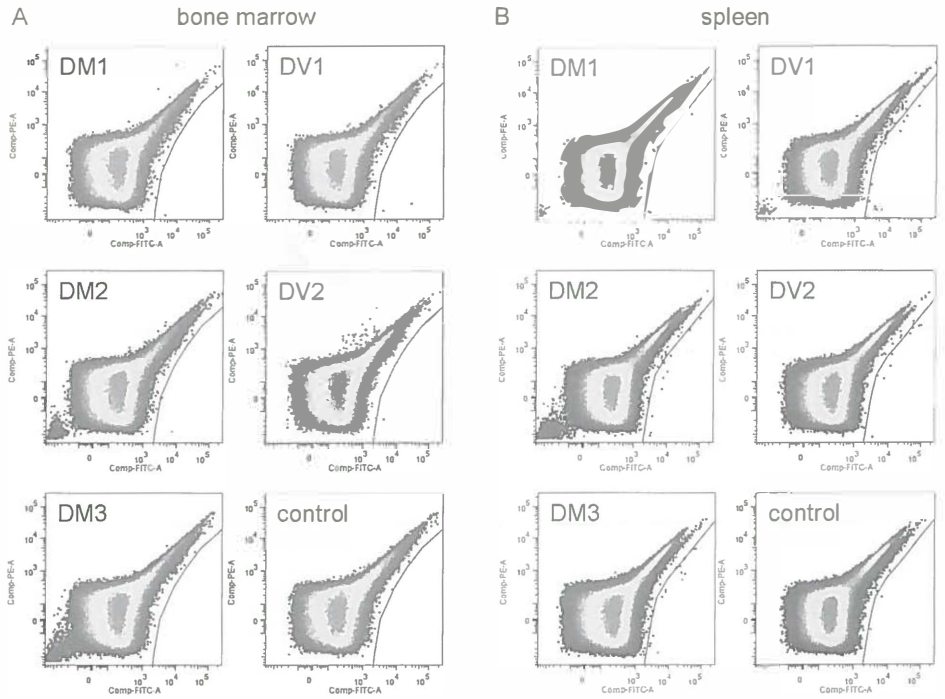

Figure 4: Detection of GFP+ MSCs in bone marrow and spleen. *FACS-plots of single cell suspensions from bone marrow (A) and spleen tissues (B) 24 hours after intravenous injection of GFP-labelled MSCs in mice. Mice that were not injected with MSCs served as control (control). Mice were inoculated with Daudi-Mock tumors (DM) or Daudi-VEGF tumors (DV).*

Accumulation in the lungs is a specific feature of MSCs.

As a control, GFP+ CD34+ cord blood cells were intravenously injected to compare accumulation of these cells in the lungs with that of MSCs. FACS analysis showed the presence of low numbers of GFP+ cells in the lungs in four out of six mice, 24 hours after injection [range 0-24 cells per 10^6 events]. In addition, 8-11 GFP+ cells with high signal intensity could be clearly detected in the bone marrow by FACS analysis (figure 5). The number of GFP+ cells in the bone marrow increased 7 to 12-fold by day 7, indicating engraftment and subsequent proliferation of CD34+ cells in the bone marrow. These results indicate very specific homing of CD34+ cells to the bone marrow without massive accumulation in the lungs.

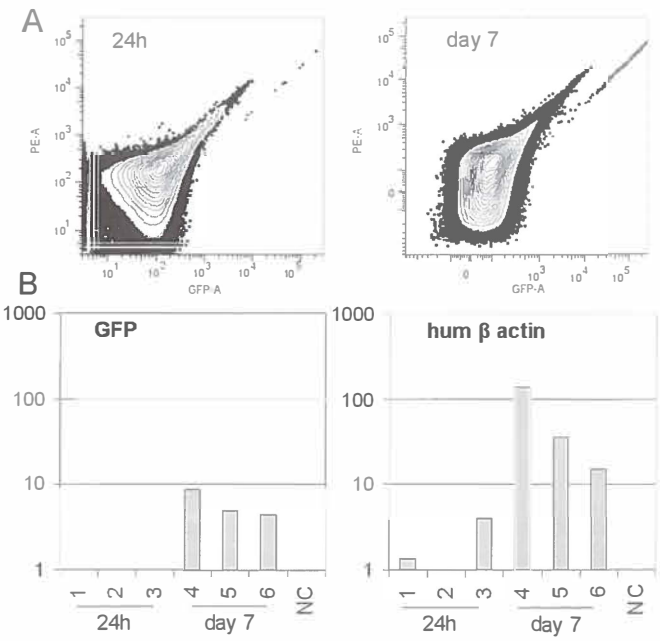

Figure 5: Detection of GFP+ CD34+ cord blood cells in the bone marrow. *A) Representative FACS-plots of single cell suspensions from bone marrow 24 hours and 7 days after intravenous injection of GFP-labelled CD34+ cord blood cells in mice. B) Relative GFP expression and relative human β-actin expression in bone marrow from CD34+ injected mice as assessed by QRT-PCR 24 hours and 7 days after intravenous injection of GFP+ CD34+ cells. Expression levels were normalized to mouse β-actin expression to correct for the RNA content, and compared to background levels (Ct-value: GFP 34.78; β-actin 34.26) using corresponding tissues from control mice that were not injected with MSCs (NC).*

Discussion

In this study, we assessed whether VEGF-A functions as a chemoattractant for circulating human BM-derived MSCs *in vivo*. Our data indicate that intravenously injected MSCs do not directly home to subcutaneous lymphoma tumors in our model, and that tumor VEGF-A does not enhance homing of circulating human BM-derived MSCs to tumors.

We used different techniques to measure the presence of GFP+ MSCs in the tumor, including FACS analysis, quantitative real time (QRT)-PCR and

immunohistochemistry. By FACS, 2 million events were counted in total for each tissue sample, resulting in a sensitivity of less than 10 positive events that can be detected out of $10^{\wedge 6}$ total events. We used QRT-PCR as a control to confirm the presence of GFP-labelled cells that were detected by FACS analysis. PCR has previously been used to detect relatively small proportions of labelled cells (0.1-1%) in tumor tissue (25). Immunohistochemistry is least sensitive in the detection of sporadic GFP+ cells. In our study, using FACS analysis (most sensitive technique), we did not detect GFP+ cells in 4 out of 6 tumors. In 2 tumors, we detected 5 and 2 GFP-positive events, however, the GFP expression level of these potentially GFP-positive cells was low and close to the border of the gate. GFP mRNA expression could not be detected in any tumor by QRT-PCR. In contrast, in the lungs, we observed obvious GFP-staining in the lungs of all mice that were injected with GFF+ MSCs using immunohistochemistry (least sensitive technique), proving the presence of MSCs in the lungs 24 hours after injection of the cells. As a control, GFP+ CD34+ cord blood cells were only detected in low numbers in the lungs of some mice, indicating that accumulation in the lungs is a specific feature of MSCs.

MSCs are able to migrate *in vitro* in response to a large set of chemotactic factors, including both chemokines and growth factors (26). In tumors, expression of vascular endothelial growth factor A (VEGF-A) is often increased in response to low oxygen concentrations and subsequent upregulation of the transcription factor HIF 1α (17). Our *in vitro* results show that VEGF-A functions as a chemo-attractant for MSCs underscoring earlier data from Beckermann et al. (17). Migration of MSCs in response to VEGF-A is proposed to be mediated by the platelet-derived growth factor receptor, since MSCs lack expression of VEGF-receptors (24;27). Although our *in vitro* experiments clearly indicate that MSCs possess tropism towards the chemoattractant VEGF-A, intravenously injected MSCs were not detected in subcutaneously implanted lymphoma tumors overexpressing VEGF-A *in vivo*. An explanation could be that all injected MSCs accumulated in the lung vascular bed, without any MSCs passing through. However, we did detect very low numbers of MSCs in the spleen by FACS, suggesting that MSCs can pass through the lung vascular bed. Thus, our data indicate that MSCs do not possess specific tropism for lymphoma tumors in our model, even not when the chemoattractant VEGF-A is overexpressed by the tumor.

In various animal models, circulating MSCs following intravenous infusion have been reported to engraft and persist long-term in a wide range of tissues, including bone marrow and lung (28-30). In the context of cancer, intravenously injected MSCs have been demonstrated to engraft in (metastatic) tumors located in the lungs and participate in tumor stroma formation, suggesting that MSCs are a potential source of stromal cells (31). Our data, however, question the proposed homing specificity of MSCs to (metastatic) tumors located in the lungs since our results indicate that most of the circulating MSCs are aspecifically filtered out of the circulation by the lung vascular bed (32). Once stuck, the tumor in the lungs may just provide the best microenvironment for the MSCs to survive and proliferate.

Few studies have reported engraftment (defined as growth of transplanted cells within a recipient tissue) of MSCs or MSC-derived cells in subcutaneous tumors after intravenous injection of MSCs (25;33-36). In NCr-nu mice i.v. injected with 5 doses of 1 million human MSCs over a 20-day period, Studeny et al. (36) detected MSC-derived fibroblasts in s.c. A375SM melanoma tumors by immunohistochemistry using a mouse-anti-human AS02 antibody specific for MSCs and not for the A375SM tumor cells. However, appropriate control stainings (tumors from mice not injected with MSCs, and IgG controls) were not shown in this paper, and acquisition of the antibody-directed fibroblast marker by the tumor cells *in vivo* can be questioned. In addition, Kucerova et al. reported engraftment of i.v. injected adipose tissue-derived MSCs in subcutaneous HT29 adenocarcinoma tumors (25) and A375 melanoma tumors (35), and Dwyer et al. (33) reported engraftment of i.v. injected MSCs in subcutaneous T47D breast tumors. Although these studies show long-term engraftment (>3 days after i.v. injection) of MSCs in subcutaneous tumors, our data indicate that, at least in our model, short-term homing of MSCs to tumors does not take place within 24 hours after i.v. injection. Our data indicate that MSCs predominantly accumulate non-specifically in the lungs rather than specifically home to the tumor micro-environment. We propose that the engraftment of MSCs in subcutaneous tumors as reported in the above mentioned studies is likely due to redistribution and secondary homing of MSCs that first have been accumulated in the lungs.

Conclusion

In conclusion, our data indicate that intravenously injected MSCs do not directly home to subcutaneous tumors in our model, and that tumor VEGF-A does not enhance homing of circulating human BM-derived MSCs to tumors. Our results suggest that spontaneous migration and direct homing of bone marrow-derived MSCs to tumors may be an uncommon event, since most of the MSCs will be filtered out of the bloodstream by the lung vascular bed.

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Chapter 6

VEGF-A promotes lymphoma tumour growth by activation of STAT proteins and inhibition of $p27^{KIP1}$ via paracrine mechanisms

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Abstract

Increased levels of circulating VEGF-A have been demonstrated in patients with non-Hodgkin lymphoma (NHL), and are associated with progressive disease and poor clinical outcome. We investigated the role of VEGF-A in lymphoma tumour growth on a molecular level in order to identify the mechanism of VEGF-A-promoted tumour growth and to identify potential targets for therapy. We used a model in which Daudi (human Burkitt lymphoma) tumour cells were transduced with VEGF-A165 or an empty vector (negative control) and subcutaneously injected in NOD/SCID mice. Tumour weight of tumours overexpressing VEGF-A was increased 4-fold compared to control tumours ($p<0.0001$), whereas no in vitro growth advantage was demonstrated upon VEGF-A overexpression. VEGF-A-tumours were associated with increased microvessel densities ($p=0.004$) and increased tumour cell proliferation (Ki67; $p<0.001$) compared to control tumours. VEGF-A-tumours were characterized by upregulation of phosphorylated STAT-4 and STAT-6 and downregulation of phospho-p27KIP1, a crucial cell cycle inhibitor ($p<0.05$). This was accompanied by increased levels of phosphorylated receptor tyrosine kinases, including EGFR (ErbB2 and ErbB4, $p<0.05$), an upstream regulator of STAT proteins. We demonstrated that various mouse-derived cytokines produced by mouse-derived tumour stromal cells are upregulated in VEGF-A-tumours compared to control tumours ($p<0.05$). These results indicate an important role for the tumour microenvironment in paracrine promotion of lymphoma tumour growth in response to tumour-derived VEGF-A. In conclusion, lymphoma-derived VEGF-A promoted lymphoma tumour growth in a paracrine loop by activation of tumour stromal cells. Our study reveals VEGF-A and STAT proteins as potential additional targets in the treatment of lymphoma.

Introduction

In non-Hodgkin lymphoma (NHL), vascular endothelial growth factor A (VEGF-A) expression has been identified in follicular B-cell lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), peripheral T-cell lymphoma (1;2) and mantle cell lymphoma (3). Increased levels of circulating VEGF-A have been demonstrated in patients with NHL (4-6), and have been associated with progressive disease and poor clinical outcomes (6-8). VEGF-A is the most important pro-angiogenic factor involved in normal and pathologic angiogenesis. In the setting of NHL, increased vessel density has been found in lymph node biopsies of patients with NHL compared to benign lymphadenopathies, and increased MVD has been related to the histological malignancy grade of the lymphoma sub-type (9). Also, an association between high MVD and decreased overall survival has been found in diffuse large B-cell lymphoma (DLBCL) (10).

NHL cells have been shown to secrete VEGF-A, and a strong correlation has been found between the amount of VEGF-A produced *in vitro* by different types of human lymphoma cell lines and the onset of tumour growth *in vivo* in NOD/SCID mice. VEGF-A production of these lymphoma tumour cell lines correlated with the frequency of endothelial cells in the tumours and inversely correlated with the frequency of apoptotic tumour cells (11). NHL cells have been shown to express VEGF-receptors (VEGFR), suggesting the existence of autocrine and paracrine pathways in lymphoma tumour growth and progression (12). These data suggest an important role for VEGF-A in lymphoma tumour growth and progression. However, the mechanism is still unclear. Understanding how VEGF-A influences lymphoma tumour growth on a molecular level could open new ways towards additional therapeutic strategies in the treatment of lymphoma.

In this study, we investigated the mechanism by which VEGF-A promotes lymphoma tumour growth. Using a xenograft mouse model with a human Burkitt lymphoma cell line transduced with VEGF-A165 or an empty vector, we demonstrate that VEGF-A significantly increased lymphoma tumour growth and tumour vessel formation. Our results demonstrate that lymphoma-derived VEGF-A stimulates tumour stromal cells to produce various cytokines resulting in paracrine activation of STAT proteins and inhibition of $p27^{KIP1}$. Our study reveals VEGF-A and STAT proteins as potential

additional targets in the treatment of lymphoma and underlines the importance of targeting the tumour microenvironment in the treatment of lymphoma.

Materials and Methods

Generation of the tumour cell lines - Daudi (human Burkitt lymphoma) cells (13) were cultured in Iscove's media supplemented with 2 mM L-glutamine, 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM ß-mercaptoethanol. Stably transduced Daudi-VEGF and Daudi-MOCK cell lines were generated using retroviral supernatants produced by 293T cells after cofransfection of packaging plasmid pCIAmpho and reporter plasmid pMSCV-VEGF-A-NGFR and pMSCV-NGFR, respectively. Retroviral supernatants were passed through a 0.45 µm filter before addition to the Daudi cells. Daudi cells were incubated with retroviral supernatant supplemented with 8 µg/ml polybrene for 16 hours in two consecutive transduction rounds. NGFR-positive Daudi cells were sorted using MoFlo XPD Cell Sorter to obtain a purity of 99 ± 1% transduced cells. The functionality of secreted VEGF-A from transduced cells was assessed by adding its supernatant to HUVEC and to quantify the expression level of the VEGF-A responsive gene EGR3 in HUVEC using real-time PCR as described in detail by Liu et al. (14). Potential differences in cell growth between the generated cell lines were assessed by culturing Daudi-MOCK and Daudi-VEGF cell lines in duplicate at a density of $0.7x10^6$ cells per ml for 20 consecutive days by passing and counting the cells every day. Cell viability was checked by Trypanblue exclusion. Cell growth was also checked by a WST assay (Roche, Woerden, The Netherlands) according to the protocol provided.

FACS analysis - VEGFR expression on Daudi tumor cells was evaluated using direct immuno-fluorescence. Cell suspensions (100 µl) were stained with 5 µl mouse-anti-human VEGFR-1 (clone 49560), mouse-anti-human VEGFR-3-APC (clone 54733) (R&D Systems, Abingdon, UK), and mouse-anti-human VEGFR-2 clone KDR-1 (Sigma, Munchen, Germany) in PBS/1%BSA for 30 min at RT. For VEGFR-1- and VEGFR-2 staining a rabbit-IgG1- anti-mouse-PE secondary antibody (DAKO AS, Glostrup, Denmark) was used. For intracellular staining cells were fixed in 4%

paraformaldehyde for 20 min and permeabilized in PBS/0.3%TritonX100 for 10 min previous to adding the primary antibodies. After staining, cells were washed with PBS/EDTA and resuspended in 300 µl PBS/EDTA before FACS analysis. Data were analysed by the Winlist software (Verity Software).

In vivo model - Non-obese diabetic–severe combined immunodeficient (NOD/SCID) mice (Charles River, Maastricht, The Netherlands) were bred and maintained in a pathogen-free environment at the Central Animal Facility, University of Groningen. All procedures involving animals were performed in accordance with local ethical animal laws and policies. During the experiment, mice were kept under laminar flow conditions. Eight week old mice were sublethally irradiated (2 Gy) and were subcutaneously injected with 10 x 10^6 Daudi tumor cell suspensions (100 µl in phosphate-buffered saline (PBS)) at their right flanks. Mice were injected with Daudi-MOCK (n=9) or Daudi-VEGF-A cells (n=9). Mice were evaluated for tumor growth every 2 days. Mice were sacrificed 11, 14 or 17 days after tumor inoculation. Tumors were removed, embedded in TissueTek (Sakura Finetek Europe, Zoeterwoude, The Netherlands), snapfrozen in melting isopentane and stored at -80°C till later use. Blood samples were taken and plasma was isolated after centrifugation of the blood and stored at -80°C till later use. Plasma VEGF-A levels were determined using a commercially available ELISA (Quantikine human VEGF-A Immunoassay, R&D Systems, Abingdon, UK) according to the protocol provided.

QRT-PCR - RNA was isolated from *ex vivo* tumors or tumor cell lines, using Machery Nagel RNA isolation kit (Machery Nagel, Düren, Germany), according to the protocol provided. cDNA was prepared and amplified using iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands) on a MyIQ thermocycler (Bio-Rad, Hercules, CA, USA) and quantified using MyIQ software (VEGFA and β-actin) or by conventional PCR (EGF and IL-4R). Gene expression was assessed using the following primer combinations (all from Invitrogen, Breda, The Netherlands): VEGF-A forward AAGGAGGAGGGCAGAATCAT, reverse CCAGGCCCTCGTCATTG; β-actin forward GCTGTGCTACGTCGCCCTG, reverse GGAGGAGCTGGAAGCAGCC; IL-4R forward ACACCTGGAGGAAGTAGAAC, reverse ACCGCATGTACAAACTCCTG; EGF forward GGACAGACAGAGCGAAATC, reverse GAGCTGGCTATAACCAGAC.

Immunohistochemical staining for micro vessel density and proliferation - Fresh serial tumor sections (4-µm) were thaw mounted on tissue-coated glass slides (Star Frost, Waldemar Knittel, Germany). Air-dried sections were fixed in acetone, blocked for endogenous peroxidase with 0.25% H_2O_2, and stained overnight with rat anti-mouse CD31 (1:100; Invitrogen, Gibco, Breda, The Netherlands) or goat polyclonal Ki67 (1:200; Santa Cruz, Heidelberg, Germany) diluted in PBS/1% BSA. An appropriate biotin-labelled secondary antibody (DAKO AS, Glostrup, Denmark) was used, followed by amplification with streptavidin ABComplex/HRPO (DAKO AS, Glostrup, Denmark) and subsequent addition of 3-amino-9-ethylcarbazole substrate for peroxidase (Sigma, Munchen, Germany). Sections were counterstained with hematoxylin. Negative controls were incubated with non-specific IgG as the primary antibody. Vessel number was assessed using light microscopy in areas of the slide containing the highest numbers of CD31-positive small blood vessels (including sinusoids and capillaries), as previously described (15). Proliferation was evaluated by determining the percentage of Ki67-positive cells by counting Ki67-positive cells in three random fields using light microscopy at 400x magnification.

Proteome profiler arrays - Mouse specific cytokine levels and human specific phospho-kinase levels in Daudi- MOCK (n=4) and Daudi-VEGF tumors (n=4) were determined using a mouse cytokine array panel A kit (40 cytokines), a human phospho-RTK array kit (42 RTKs) and a human phospho-kinase array kit (46 kinases) (all from R&D Systems, Abingdon, UK) according to the protocols provided. 400 µg protein was applied on each array. Spot densities were quantified with Scanalyze software (http://rana.lbl.gov/EisenSoftware.htm) and exported to Microsoft Excel. Spot densities were corrected for the individual background to diminish interarray variances.

Statistical analysis - All values in the figures represent mean ± SD. The relevant data sets were compared by unpaired Student's t test (tumour weight, MVD) or Mann-Whitney-U test (proteome profiler arrays, Ki67) using SPSS software. P-values <0.05 were considered statistically significant.

Results

Functional VEGF-A overexpression by the Daudi-VEGF cell line.

A 92.6-fold induction of VEGF-A expression was achieved after stable transduction of Daudi tumor cells with the VEGF-A gene (figure 1A). Functionality of the produced VEGF-A was confirmed by addition of supernatant of Daudi-VEGF-A or Daudi-MOCK to endothelial cells (HUVEC). Expression of the VEGF-A-responsive gene EGR-3 was 7.7-fold upregulated in HUVEC cells after incubation with DV-conditioned medium, whereas unconditioned control medium had no effect on EGR-3 expression in HUVEC cells (figure 1B). These results indicate that the Daudi-VEGF cell line produced and secreted functional VEGF-A-protein *in vitro*.

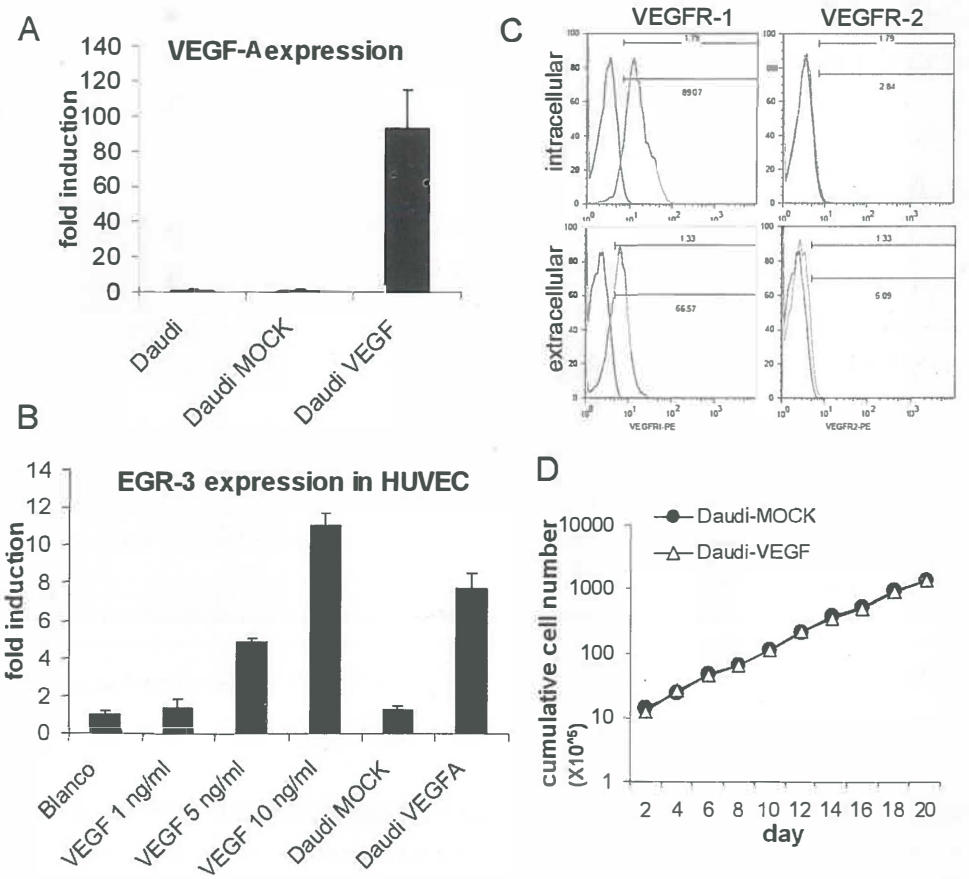

Figure 1: VEGF-A and VEGF-receptor expression in Daudi tumour cells. *A) Fold induction of VEGF-A mRNA expression in VEGF-A-transduced and empty vector (MOCK)-transduced Daudi tumor cells compared to untransduced Daudi tumor cells. B) Induction of mRNA expression of the VEGF-A responsive gene EGR3 in HUVEC after incubation with conditioned medium from VEGF-A-transduced and empty vector (MOCK)-transduced Daudi tumor cells compared to control medium (blanco). C) Extracellular and intracellular expression of VEGFR 1 and VEGFR-2 on Daudi tumor cells. D) Growth curves of VEGF-A-transduced and empty vector (MOCK)-transduced Daudi tumor cells. The Y-axis represents cumulative cell numbers.*

FACS analysis showed that Daudi tumor cells do not express VEGFR-2, whereas the majority of cells expressed VEGFR-1 (65% extracellular and 87% intracellular; figure 1C) and VEGFR-3 (89% extracellular and 7% intracellular; data not shown). Results were similar for Daudi-MOCK and Daudi-VEGF cell lines. Growth and viability of Daudi-VEGF and Daudi-MOCK cells was similar (figure 1D), which was confirmed by a WST assay (data not shown), indicating that potential autocrine stimulation of tumor cell growth by VEGF-A is not detectable *in vitro*.

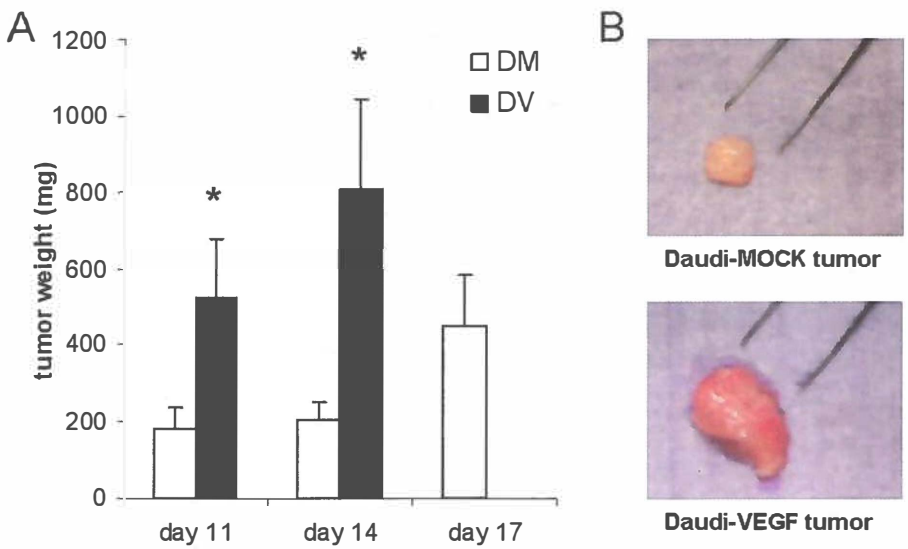

Figure 2: VEGF-A overexpression in lymphoma tumours increases tumour growth. *A) Tumor weight (mg) of Daudi-VEGF (DV) and Daudi-MOCK (DM) tumors 11, 14, and 17 days after in vivo inoculation. *p<0.05. B) Two representative pictures of a Daudi-MOCK tumor (left) and a Daudi-VEGF tumor (right) excised on day 14.*

VEGF-A over-expression in lymphoma tumours promotes tumour growth and tumour vessel formation. Daudi-MOCK and Daudi-VEGF-A tumor cells were inoculated subcutaneously in mice using a NOD/SCID xenograft mouse model. Tumor weight of tumors overexpressing VEGF-A (DV-tumors) was significantly increased compared to control tumors (DM-tumors) (tumor weight day 14; DV 807 ± 240 mg vs. DM 205 ± 44 mg, $p<0.0001$) (figure 2). Tumor micro vessel density (MVD) was significantly increased in DV-tumors compared to DM-tumors (day 14; DV 57.7 ± 15.5/HPF vs. DM 14.7 ± 1.0/HPF, $p=0.004$) (figure 3A). Tumors overexpressing VEGF-A were characterized by large nodules of extended networks of irregular, elongated blood vessels, while DM-tumors showed fewer blood vessels that were smaller and rounder than in VEGF-A-tumors (figure 3B). VEGF-A mRNA-expression quantified by QRT-PCR was upregulated 35.1-fold in DV-tumors compared to DM-tumors (data not shown). Moreover, VEGF-A-protein could be detected in plasma of mice bearing DV-tumors (mean 282.0 ± 147.1 pg/ml), whereas plasma VEGF-A in mice bearing DM-tumors was undetectable (<10 pg/ml) (data not shown). Together, these data show that VEGF-A overexpression in Daudi lymphoma tumors is associated with increased tumor growth and vascularization.

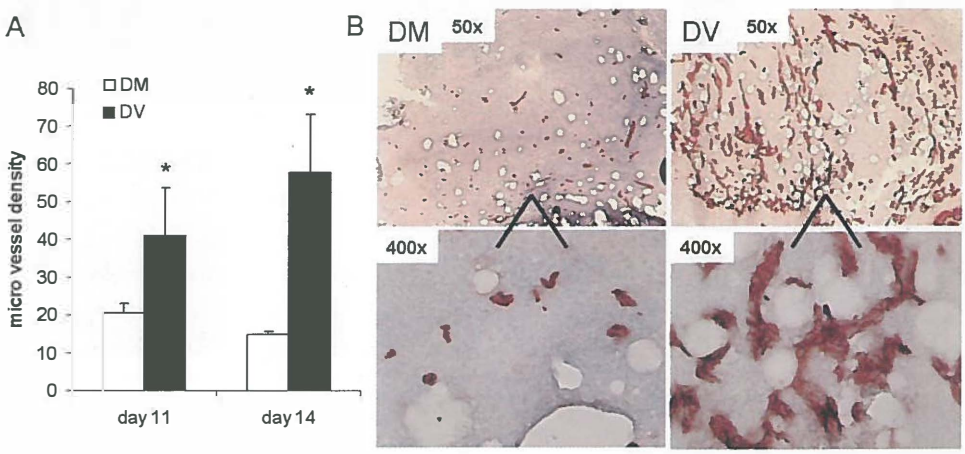

Figure 3: Micro vessel density. *A) Micro vessel densities (MVD) scored in Daudi-MOCK tumors (DM) and Daudi-VEGF tumors (DV) that were excised 11 and 14 days after in vivo inoculation. *p<0.05. Graph represents the mean MVD ± stdev. B) Representative pictures of CD31-stained tumor sections of a DM-tumor and a DV-tumor at 50x and 400x magnification.*

Activation of STAT-4 and 6, and inhibition of p27^{KIP1} in VEGF-A-tumours.

Ki67-staining on tumor sections showed an increased fraction of proliferative cells in tumors overexpressing VEGF-A (DV-tumors) compared to control tumors (DM-tumors) (figure 4A and B). Using proteome profiler arrays, we assessed whether the increased proliferative profile of DV-tumors would be reflected in altered intracellular signalling compared to DM-tumors. Levels of 7 intracellular phosphorylated kinases were significantly increased in DV-tumors compared to DM-tumors, including STAT-4, STAT-6, eNOS, Pyk2, Paxillin, c-Jun and PLCγ-1 ($p<0.05$) (figure 4C and D). In contrast, levels of phosphorylated p27^{KIP1} (T198 and T157) were significantly decreased in VEGF-A overexpressing tumors compared to control tumors ($p<0.05$). p27^{KIP1} is an important cell cycle inhibitor (16), and STAT proteins have been reported to regulate p27^{KIP1} expression (17), indicating an important role for STAT proteins and p27^{KIP1} in promoting lymphoma tumor growth as a result of VEGF-A overexpression.

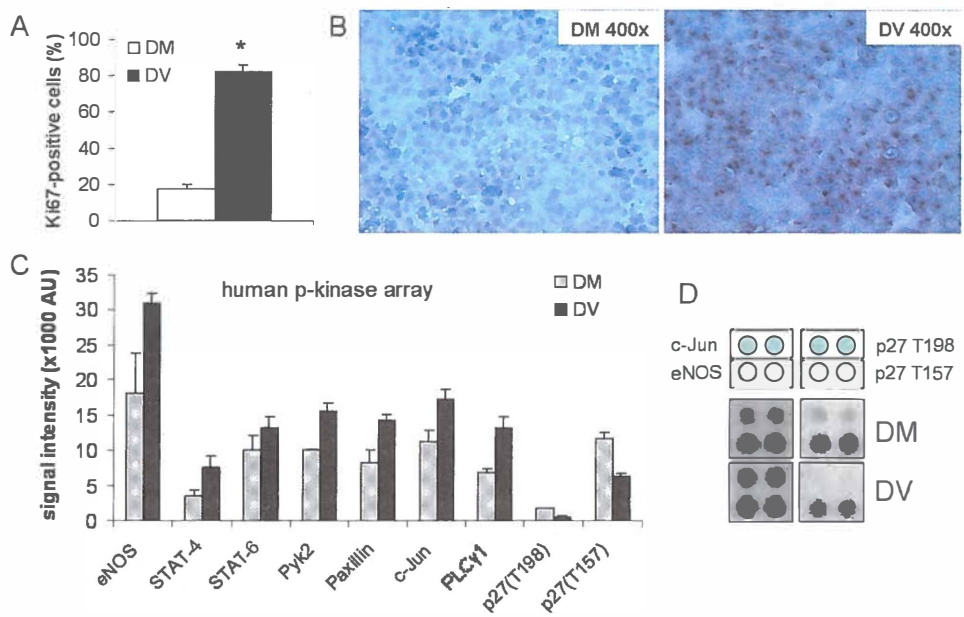

Figure 4: Proliferation index and phospho-kinase arrays. *A) Graph represents the mean percentage of Ki67-positive cells ± stdev in 3 DM-tumors and DV-tumors (day 14). *p<0.001. B) Representative pictures of Ki67-stained sections of a DM-tumor and a DV-tumor at 400x magnification. C) Human specific phospho-intracellular kinase array. Graph represents the mean signal intensity (AU) ± stdev of significantly upregulated or downregulated phospho-kinases (p<0.05) in DV-tumors and DM-tumors (day 14). D) Close up of array expression of c-Jun, eNOS and p27 in a representative example of a DV-tumor and a DM-tumor.*

Paracrine mechanisms play important roles in VEGF-A-promoted tumour growth. Although Daudi-VEGFA tumor cells had no growth advantage over Daudi-Mock tumor cells *in vitro,* the *in vivo* growth benefit of tumors in response to VEGFA overexpression was significant. Moreover, no VEGFR2 expression was detected on Daudi-Mock and Daudi-VEGFA cells. The increased fraction of mouse-derived stromal cells, including endothelial cells, in VEGF-A overexpressing tumors may be associated with increased production of growth factors and cytokines in these tumors. In turn, these growth factors may induce receptor-signalling in tumor cells resulting in paracrine promotion of tumor cell proliferation. Indeed, we found that levels of 27 mouse-derived cytokines were significantly increased in VEGF-A overexpressing tumors (DV-tumors) compared to control tumors (DM-tumors) ($p<0.05$) (figure 5A and B). Upregulated cytokines were many interleukins (IL-1ra, IL-16, IL-1a, IL-23, IL-17, IL-27, IL-7, IL-1b, IL-4, IL-3, IL-2, and IL-10); chemokines (MIG/CXCL-9, IP-10/CXCL-10, RANTES/CCL-5, MIP-2, MIP-1b/CCL-4, I-309 (CCL-1/TCA-3), I-TAC/CXCL-11, and MIP-1a/CCL-3); growth factors (M-CSF, G-CSF, and GM-CSF); and other cytokines (IFNγ, KC, TREM-1, and C5a). In accordance with literature many of these cytokines can augment tumor cell proliferation by activation of STAT proteins. For example, IL-4-induced proliferation is mediated by STAT-6 (17). IL-4 binds to the IL-4-receptor (IL-4R), which is known to be expressed by Daudi tumor cells (18). We confirmed IL-4R expression on Daudi tumor cells by conventional PCR (data not shown). Together, these data show that stroma-derived cytokines are upregulated in tumors overexpressing VEGF-A, suggesting that VEGF-A exerts its effect on lymphoma tumor growth via a paracrine loop.

Next, we validated this hypothesis by investigating whether the upregulation of cytokines in DV-tumors is associated with alterations in the activity of receptor tyrosine kinase signalling. In DV-tumors, levels of phosphorylated ErbB-4 (EGFR), ErbB-2 (EGFR), FGFR-2a, EphB-1, MuSK and InsulinR were significantly increased compared to control tumors ($p<0.05$) (figure 5C). Ligand-binding to EGFR is known to activate STAT proteins (19). Using conventional PCR we confirmed expression of EGF (EGFR-ligand) in Daudi tumour cells (data not shown). Levels of other phosphorylated receptors were significantly decreased in DV-tumor compared to DM-tumors (EphB-2, ROR-1, and FGFR-3) (figure 5C). Levels of phosphorylated VEGF-

receptors were not different in DV-tumors compared to control tumors (data not shown).

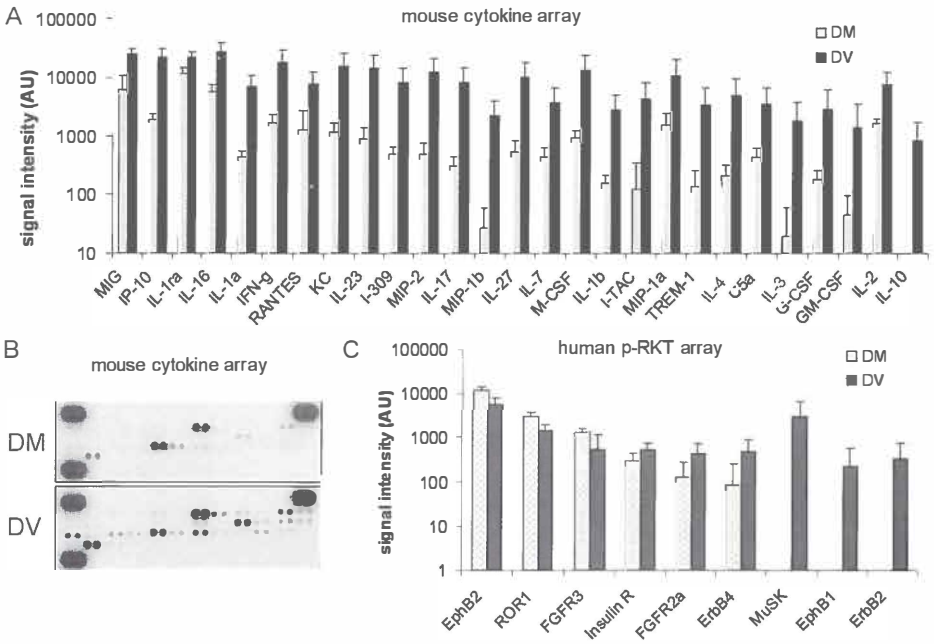

Figure 5 (next page): Proteome profiler arrays: *A) Mouse specific cytokine arrays, and C) Human specific phospho-receptor tyrosine kinase (RTK) arrays. Graphs represent the mean signal intensity (AU) ± stdev of significantly upregulated or downregulated cytokines (A), or phospho-RTKs (C) ($p<0.05$) in DV-tumors and DM-tumors (day 14). B) Representative example of a cytokine array of a DV-tumor and a DM-tumor.*

Discussion

In this study, we showed that VEGF-A overexpression by Daudi lymphoma tumors significantly increased lymphoma tumor growth *in vivo*. This was accompanied by increased tumor angiogenesis and an increased fraction of proliferating tumor cells. VEGF-A-tumors were characterized by upregulation of phosphorylated STAT-4 and STAT-6 and downregulation of phosphorylated $p27^{KIP1}$, a crucial cell cycle inhibitor. This was accompanied by increased levels of phosphorylated receptor tyrosine kinases, including EGFR, a known upstream regulator of STAT proteins. In addition,

we demonstrated that tumor-derived VEGF-A activates mouse-derived stromal cells in a way that mouse-derived cytokines are upregulated in VEGF-A-tumors compared to control tumors. All together, our results indicate an important role for the tumor microenvironment in paracrine promotion of lymphoma tumor growth in response to tumor-derived VEGF-A, which is mediated by STAT proteins and $p27^{KIP1}$. Figure 6 shows a provisional scheme of the pathway of VEGF-A-promoted tumor growth.

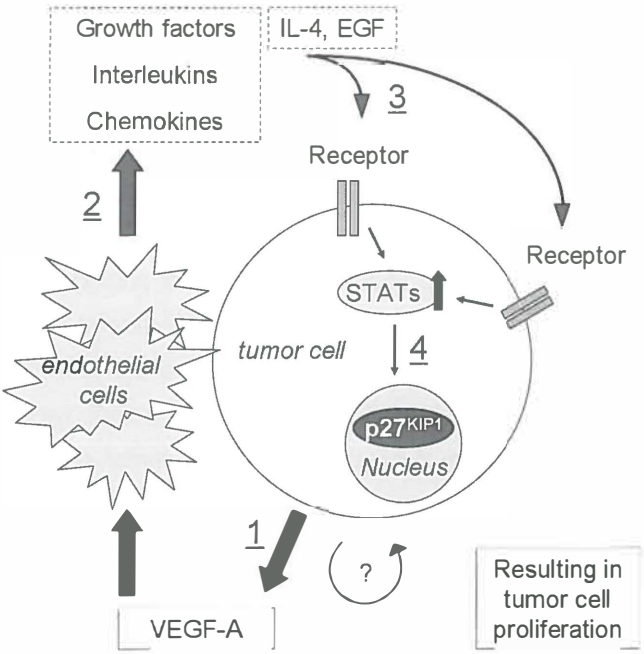

Figure 6: Provisional scheme on the pathways involved in VEGF-A-promoted tumour growth. *VEGF-A production in the tumor results in an increased fraction of endothelial cells in the tumor microenvironment (1). These tumor endothelial cells produce a variety of factors (growth factors, interleukins and chemokines) (2) that act on tumor cells by binding to receptors that are present on the tumor cell membrane (3). Activated receptors induce signal transduction pathways (4), which results in activation of STAT proteins and inhibition of p27KIP1. This, in turn, results in increased tumor cell proliferation, which may explain the increased growth of VEGF-A overexpressing tumors in our model.*

In patients with NHL, increased levels of circulating VEGF-A have been associated with progressive disease (6). In our model, VEGF-A overexpression by the tumor resulted in detectable levels of plasma VEGF-A, and was associated with increased tumor growth, underlining the feasibility of our model to study human lymphoma

growth. In this study, tumors overexpressing VEGF-A were associated with increased levels of phosphorylated STAT proteins and decreased levels of phosphorylated $p27^{KIP1}$. $p27^{KIP1}$ is a target gene of STAT proteins, and the expression of $p27^{KIP1}$ in lymphomas is inversely related with proliferation, except for mantle cell type lymphoma (20). Lack of $p27^{KIP1}$ in $p27^{KIP1}$-knockout mice has been associated with lymphoproliferative dysregulation (21;22). Moreover, loss of $p27^{KIP1}$ is indicative of poor outcome (shorter survival) in non-hodgkin lymphoma (NHL) patients (23). Recently, Zhao et al. reported a negative relation between $p27^{KIP1}$ and Ki67 in NHL patients; low levels of $p27^{KIP1}$ were associated with high levels of Ki67 after immunohistochemical staining of tumor sections (24). Interestingly, we also found increased Ki67 staining in tumors overexpressing VEGF-A. To our knowledge, our study is the first to show that the effect of VEGF-A on lymphoma growth is mediated by tumor STAT proteins and $p27^{KIP1}$.

VEGF-A is known to be the most important mediator of angiogenesis. In our study, we observed that VEGF-A overexpression in the tumor indeed was associated with significantly increased micro vessel density (MVD). In general, blood vessels not only supply oxygen and nutrients to the tumor cells, but also provide various endothelium-derived growth factors that are of potential benefit for tumor cells. In our study, we found many stromal (mouse) derived chemokines, cytokines (such as G-CSF and GM-CSF) and many interleukins (such as IL-4 and IL-6) to be upregulated in response to VEGF-A overexpression in the tumor. Exposure of human endothelial cells to VEGF-A, has been shown to result in increased production of cytokines, including IL6, G-CSF, GM-CSF and M-CSF (25;26). Besides endothelial cells other tumor stromal cells also can contribute to cytokine, interleukin, and growth factor production upon VEGF-A stimulation. These stroma-derived cytokines, in turn, can act as growth factors for lymphoid malignant cells, suggesting paracrine interactions between hematopoietic malignant cells and the newly generated endothelium (25;26). In addition, interleukins are mainly described in relation to inflammation, a process that promotes progressive tumor growth and angiogenesis (27). Together, these results indicate an important role for the tumor stroma in promoting lymphoma tumor growth via paracrine mechanisms. Besides paracrine mechanisms, VEGF-A has been shown to contribute to leukaemia and lymphoma tumour growth by autocrine stimulation (12;28;29). In our study, however, despite a ~90-fold induction

of VEGF-A in the Daudi cell line overexpressing VEGF-A, *in vitro* growth of these cells was similar to that of control Daudi cells. Moreover, Daudi cells lacked expression of VEGFR-2, suggesting that the VEGF-A-promoted tumor growth observed in our *in vivo* model is not the result of autocrine mechanisms.

Despite improvement of treatment strategies in the last decades, about 20,3% of children and about 31% of adults with NHL still die from their diseases within 5 years after diagnose (1), underlining the relevance of additional research to improve lymphoma treatment strategies. Our study reveals VEGF-A, STAT proteins and $p27^{KIP1}$ as potential additional targets in the treatment of Burkitt lymphoma. Interestingly, the tyrosine kinase inhibitor AGL2592 has been shown to induce persistent growth arrest and apoptosis in human NHL cell lines by inhibition of STAT3 phosphorylation (30). Moreover, inhibition of STAT3 phosphorylation sensitized the NHL cell line 2F7 to a wide range of chemotherapeutic drugs, demonstrating the potential of inhibiting STAT pathways to overcome drug resistance in NHL (31). Recently, single and multicenter phase II clinical studies showed that the proteasome inhibitor bortezomib (which allows preventing, among others, the degradation of $p27^{KIP1}$, thereby inhibiting cell cycle progression) can be used as additional drug in the treatment of mantle cell and follicular lymphoma (32-35). Inhibition of VEGF-A/VEGFR signalling in lymphoma has shown to be promising in preclinical studies (12;36;37). Moreover, in humans, results of a phase II clinical trial with bevacizumab, a monoclonal antibody against VEGF-A, has already shown well-tolerated and prolonged stabilization of disease in patients with relapsed, aggressive NHL (38). Combining conventional chemo- or immunotherapy regimens with drugs targeting the tumor microenvironment may be of benefit for the treatment of lymphoma.

Conclusion

In conclusion, we demonstrated that VEGF-A significantly increased Burkitt lymphoma tumor growth and tumor vessel formation. Our results indicate an important role for the tumor microenvironment in paracrine promotion of lymphoma tumor growth in response to tumor-derived VEGF-A, by activation of STAT proteins

and inhibition of $p27^{KIP1}$ in tumor cells. Our study reveals VEGF-A and STAT proteins as potential additional targets in the treatment of lymphoma.

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Chapter 7

Summary, general discussion and conclusion of the thesis

Summary

The aim of this thesis was to determine promotion of lymphoma tumor growth by bone marrow-derived mesenchymal stem cells (MSCs), to explore the mechanism by which MSCs would promote tumor growth, and to explore ways to inhibit the effects of MSCs on tumor growth.

In **chapter 2**, different types of bone marrow-derived cells that have been shown to contribute to tumor growth were discussed. In addition, the potential role of MSCs in modulating tumor micro-environments was discussed, as well as the potential of MSCs as cellular vehicles for selective delivery of cancer therapeutics.

In **chapter 3**, we described that we explored ways to inhibit the tumor growth-promoting effects of MSCs by testing the small molecule tyrosine kinase inhibitor PTK787/ZK 222584 (Vatalanib, PTK). We demonstrated that PTK inhibits the outgrowth of MSCs from bone marrow mononuclear cells *in vitro*. In addition, administration of PTK to MSC-cultures resulted in decreased proliferation, increased apoptosis, impaired migration and an impaired capacity to form tube-like structures. These results suggest that PTK may be useful as a medicine to target the potential tumor-growth promoting effects of MSCs.

In **chapter 4**, it was shown that the presence of MSCs in the tumor microenvironment resulted in increased tumor growth in our *in vivo* model. These tumors were associated with an increased micro vessel density, underlining the role for MSCs in promoting vessel formation. We further investigated potential direct effects of MSCs on tumor cells using an *in vitro* co-culturing system. These experiments showed that MSCs promote tumor cell growth partly by soluble factors but predominantly by direct cell-cell contact interactions. In addition, we found that MSCs can protect tumor cells against apoptosis. Interestingly, administration of PTK to co-cultures of MSCs and tumor cells abolished this protective effect of MSCs. Furthermore, in mice, treatment with PTK seemed to result in a reduction of MSC-promoted tumor growth.

Bone marrow-derived MSCs are thought to be mobilized into the peripheral blood and to home to tumors in response to factors produced by the tumor, such as vascular endothelial growth factor A (VEGFA). In **chapter 5**, we assessed whether VEGFA overexpression by the tumor functions as a chemoattractant for circulating human bone marrow-derived MSCs. We describe that intravenously injected MSCs do not directly home to subcutaneous tumors in our model. Instead, we found massive infiltration of MSCs in the lungs. Although most MSCs were filtered out of the circulation by the lungs, small numbers of MSCs were detected in the spleen, indicating that at least some MSCs passed through the lung vascular bed. The absence of detectable MSCs in the tumors indicates that MSCs lack tumor tropism in our model even when VEGFA is overexpressed by the tumor. It thus seems that, although MSCs showed migration to VEGFA in *in vitro* migration experiments, VEGFA does not function as a chemoattractant for BM-derived MSCs *in vivo*.

In **chapter 6**, we described that VEGFA production in the tumor significantly promotes lymphoma growth. This was associated with significantly enhanced tumor vessel formation. We demonstrated that various mouse-derived cytokines produced by mouse-derived tumor stromal cells were upregulated in VEGFA-tumors compared to control tumors. Our results indicate that lymphoma-derived VEGFA stimulates tumor stromal cells to produce various cytokines resulting in paracrine activation of STAT proteins and inhibition of $p27^{KIP1}$. Our study reveals VEGFA and STAT proteins as potential additional targets in the treatment of lymphoma.

General discussion and future perspective

Inhibition of bone marrow-derived MSCs

To target MSCs, we used the small molecule tyrosine kinase inhibitor PTK787/ZK 222584 (Vatalanib) (chapter 3). Our results indicate that PTK787/ZK 222584 not only inhibits tumor endothelial cells, but also inhibits proliferation and function of bone marrow-derived MSCs and/ or MSC-derived cells that may be present in the tumor microenvironment. PTK787/ZK 222584 was originally designed to target tumor vessel formation by inhibition of VEGF-receptor signalling in tumor endothelial cells (1). Results from Bergers et al. highlighted the potential significance of combined targeting of tumor-associated pericytes (which can be MSC-derived) and endothelial cells to reduce tumor growth and progression: in a mouse model of pancreatic islet cancer, it was shown that combination treatment with PDGFR-inhibitors to target PDGFR-positive pericytes (SU6668 or STI571/Gleevec) and VEGFR-inhibitors to target VEGFR-positive endothelial cells (SU5416) was more effective than treatment with single agents (2). In clinical trials, treatment of cancer patients with PTK787/ZK 222584 has already shown to be effective in reducing tumor growth by inhibiting tumor vessel formation (3;4). New evidence from clinical trials can be expected in future.

MSCs and tumor growth

Our results demonstrated that human bone marrow-derived MSCs co-injected with tumor cells at a 1:10 ratio promoted lymphoma tumor growth in NOD/SCID mice (chapter 4). The contribution of MSCs to *in vivo* tumor growth is controversial, since MSCs have been shown to both promote and inhibit tumor growth in different models (5-11). The effect of MSCs on tumor growth is even more difficult to interpret, as they can have opposite effects *in vitro* and *in vivo* (9). These contradictory findings have resulted in the statement that "MSCs in cancer can be friends or foes". The effect of MSCs on tumor growth may be dependent on the tumor cell line used (7). In addition, another critical parameter related to promotion or inhibition of *in vivo* tumor growth by MSCs is the ratio of MSCs and cancer cells. Djouad et al. showed that tumor growth (Renca tumor cells: mouse renal adenocarcinoma) was not affected by coinjection of the same amounts of MSCs and tumor cells, but was increased in the presence of

10-fold more MSCs, whereas 10-fold fewer MSCs completely abolished tumor formation (7). In this thesis, we demonstrated promotion of Burkitt lymphoma tumor growth with 10-fold fewer MSCs than tumor cells. These results indicate that Burkitt lymphoma may be relatively sensitive to the effects of bone marrow-derived MSCs compared to other tumor types.

Mechanisms of MSC-promoted tumor growth

We showed that MSCs promote tumor cell proliferation and prevent tumor cell apoptosis *in vitro* (chapter 4). These data are in accordance with earlier studies (7;9;12). Our study, however, adds up to the knowledge on the mechanism by which MSCs promote tumor growth. We determined that production of growth factors by MSCs as well as direct cell-cell contact interactions between MSCs and tumor cells are very important in the promotion of Daudi tumor cell proliferation and the prevention of apoptosis. Direct cell-cell contact may be mediated by integrins and fibronectin. MSCs express integrins and produce extracellular matrix molecules (ECM), and it was found that binding of tumor cells to integrins and ECM promotes tumor cell survival (13). In addition, on adhesion to fibronectin, tumor cell lines and primary acute myeloid leukaemia (AML) cells become resistant to spontaneous or drug-induced apoptosis (14). Further research on the identification of specific adhesion molecules involved in the enhancement of tumor cell proliferation and protection against apoptosis by MSCs is needed to discover specific MSC-tumor cell interactions that may be relevant to inhibit for future therapies.

We showed that the presence of MSCs in the tumor microenvironment was associated with increased vessel formation. MSCs have been proposed to physically contribute to tumor vessel formation by differentiation into endothelial-like cells and pericyte-like cells (15-18) and incorporation in to the vasculature *in vivo* (19-22). Although MSCs were still present in the tumor microenvironment after three weeks of *in vivo* inoculation, the number of MSCs was too small to determine the exact fate of these cells. Recent research provided evidence that MSCs can also differentiate into tumor associated fibroblasts (TAFs) (23). Results from Speath et al. suggested the involvement of the MSCs as a component of tumor associated fibrovascular networks, which included a pericytic population that contributes to microvessels as

well as a fibroblastic population that contributes to matrix remodeling and the production of tumor-stimulating paracrine factors (23). In conclusion, it will be obvious that, in whichever form they will be present in the tumor microenvironment, MSCs can biologically impact tumor progression.

Homing of MSCs to tumors

In our xenograft model, we showed that intravenously injected human bone marrow-derived MSCs do not directly home to subcutaneous lymphoma tumors within 24 hours, even not when VEGFA is overexpressed by the tumor (chapter 5). We discussed that studies regarding tumor homing of MSCs using (metastatic) pulmonary models are hard to interpret since intravenously injected MSCs accumulate unspecifically in the lung vascular bed. Recent research in non-tumor bearing mice using bioluminescence imaging confirms that intravenously delivered MSCs indeed initially reside in the lungs, then egress to the liver and spleen, and disappear over time (24;25). However, in mice bearing subcutaneous or pulmonary tumors, selective and persistent engraftment of MSCs at the tumor site was observed (24;25). Adding our results to these earlier findings, we discussed that tumor engraftment of intravenously injected MSCs in subcutaneous tumor models may be the result of redistribution and secondary homing of MSCs that first accumulated in the lungs.

Observations of MSCs persisting specifically in tumor microenvironments have encouraged investigation into using these cells as delivery vehicles for cancer therapeutics. However, the use of systemically delivered MSCs is not without any risk. In various animal models, intravenously injected MSCs have been reported to engraft and persist long-term in a wide range of tissues (26-28), indicating that homing of MSCs is not clearly dictated by the presence of tumor cells. Similarly, although it reduced accumulation of cells in the lungs, intracardiac injection of a hMSC-TERT cell line resulted in unspecific accumulation in many organs (29). The unspecific distribution pattern of systemically delivered MSCs may be problematic in the setting of MSC-based therapies since this may result in harmful side-effects in healthy tissues. Notably, in our hands, intracardiac injection of primary human MSCs into mice was lethal, probably as a result of embolisms in the brain and/ or other

organs. Others have reported death of mice after intravenous injection of MSCs via the tail vein (25). Alternative delivery strategies are under investigation. For example, regional delivery of MSC-IFN-ß by intra-arterial injection has already been shown effective in the treatment of orthotopic U87 glioma in nude mice (30).

How likely would it be that bone marrow-derived MSCs spontaneously mobilize from the bone marrow and home to tumors? To date, circulating MSCs have been detected in the adult peripheral blood of a variety of mammalian species, including humans (31-35). The estimated number of circulating MSCs is, however, extremely low and quite common to fail in detecting any of them (36;37). In mice, MSCs can be mobilized from the bone marrow into the peripheral blood by granulocyte colony stimulating factor (G-CSF) and migrate and incorporate into the myocardium after infarction (38). Moreover, spontaneous migration of bone marrow MSCs (and/ or MSC-derived cells) has been observed in a rat model of myocard infarction (39) and in a murine model of osteogenesis imperfecta (40). These data suggest that MSCs residing in the bone marrow indeed may be able to mobilize into the peripheral blood under certain circumstances. However, direct evidence of spontaneous homing of bone marrow-derived MSCs to tumors is still lacking. The optimal model to study this would be a model in which only bone marrow MSCs are labelled. After induction of tumor growth, the presence of labelled cells in the tumor would prove spontaneous tumor homing of bone marrow-derived MSCs and/ or MSC-derived cells.

VEGFA and lymphoma growth

We demonstrated that VEGFA overexpression in the tumor significantly increased Burkitt lymphoma tumor growth and tumor vessel formation (chapter 6). Our results indicate an important role for the tumor microenvironment in paracrine promotion of lymphoma tumor growth by activation of STAT proteins and inhibition of $p27^{KIP1}$ in tumor cells. $p27^{KIP1}$ is a target gene of STAT proteins, and is a negative regulator cell cycle progression. Recently, Zhao et al. reported a negative relation between $p27^{KIP1}$ and Ki67 in NHL patients; low levels of $p27^{KIP1}$ were associated with high levels of Ki67 after immunohistochemical staining of tumor sections (41). In this thesis, we also found increased Ki67 staining in both tumors overexpressing VEGFA and tumors co-injected with MSCs. Interestingly, decreased levels of phosphorylated

$p27^{KIP1}$ were also observed in tumors co-injected with MSCs (chapter 4). To our knowledge, we are the first to show that tumor-$p27^{KIP1}$ plays an important role in mediating the effects of VEGFA and MSCs on Daudi Burkitt lymphoma growth. These results suggest that $p27^{KIP1}$ and its upstream regulators may be important targets in the treatment of Burkitt lymphoma. Interestingly, in humans, preliminary results of a phase II clinical trial with bevacizumab, a monoclonal antibody against VEGFA, has already shown well-tolerated and prolonged stabilization of disease in patients with relapsed, aggressive NHL (42). Furthermore, a preclinical study has shown that inhibition of STAT3 phosphorylation sensitized the NHL cell line 2F7 to a wide range of chemotherapeutic drugs, demonstrating the potential of inhibiting STAT pathways to overcome drug resistance in NHL (43).

Conclusion

In conclusion, in this thesis, we showed that bone marrow-derived MSCs promote tumor growth in a xenograft mouse model of human Burkitt lymphoma. Our research provides more insight in the mechanism by which MSCs promote tumor growth: MSCs produce soluble factors and provide direct cell-contact interactions that promote tumor cell proliferation and reduce tumor cell apoptosis. Further research is needed to discover specific MSC-tumor cell interactions that may be relevant to inhibit for future therapies. In addition, we showed that tumor-$p27^{KIP1}$ plays an important role in mediating the environmental effects (of VEGFA and MSCs) on Daudi Burkitt lymphoma growth. Our results indicate that $p27^{KIP1}$ and its upstream regulators may be important targets in the treatment of Burkitt lymphoma. Further research is needed to test the effect of specific inhibitors on Burkitt lymphoma growth.

The use of MSCs in clinical practise can be both a risk and benefit since MSCs can home to tumors and contribute to tumor growth, while they are also being investigated as therapeutic vehicles to inhibit tumor growth. On the one hand, additional research is necessary to investigate the feasibility of using MSCs as cellular vehicles for the selective delivery of cancer therapeutics regarding unwanted side effects and bio-safety issues. On the other hand, further research is needed to get more insight into the (spontaneous) homing capacity of bone marrow-derived MSCs to different tumor types, the relative contribution of bone marrow-derived MSCs to different tumor types, and the relevance of targeting bone marrow-derived MSCs for future anti-cancer therapies. Future research regarding tumor homing of MSCs is challenging. Our study underlines the complexity of research on tumor homing of bone marrow-derived MSCs. Although bone marrow-derived MSCs seem to lack tumor tropism, their contribution to tumor growth may not be negligible. The devil is in the details!

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Chapter 8

Nederlandse samenvatting - Dutch summary

Inleiding

Non-Hodgkin Lymphoma (NHL) is een groep van kwaadaardige hematologische afwijkingen die hun oorsprong vinden in het lymfatische systeem. NHL is een ziekte van het immuunsysteem die ontstaat door de transformatie van een lymfatische voorlopercel naar een kwaadaardige kankercel. Deze kwaadaardige cellen vormen tumoren in lymfeknopen of op andere plaatsen in het lichaam, waar het immuunsysteem prominent aanwezig is, zoals de huid, de milt en het beenmerg. Lymphoma (NHL en Hodgkin lymphoma; 15%) is, na leukemie (31%) en hersentumoren (16,6%), de meest voorkomende vorm van kanker bij kinderen. Ondanks de vooruitgang in behandelingsmogelijkheden ter bestrijding van NHL, sterft nog altijd 20,3% van de kinderen en 31% van de volwassen met NHL binnen 5 jaar na diagnose.

Er zijn de laatste tijd steeds meer aanwijzingen gevonden dat de directe omgeving waarin tumorcellen zich bevinden (micro-omgeving van de tumor) een bepalende rol heeft in het ontstaan en het doorgroeien van kanker. De micro-omgeving van de tumor bestaat uit verschillende soorten stromale cellen, waaronder endotheelcellen, pericyten, gladde spiercellen en fibroblasten. Stromale cellen zijn geen passieve toeschouwers van tumorgroei maar leveren een actieve bijdrage aan de ontwikkeling en de uitbreiding van kanker. Deze stromale cellen produceren een grote variëteit aan groei factoren, chemokines en enzymen, welke allemaal kunnen bijdragen aan processen die de micro-omgeving van de tumor kunnen veranderen en welke essentieel zijn voor de groei en de uitbreiding van kanker.

Onderzoek heeft aangetoond dat endotheelcellen, die in de micro-omgeving van de tumor voorkomen, uit het beenmerg afkomstig kunnen zijn. Bovendien wordt er verondersteld dat andere stromale cellen, zoals pericyten, gladde spiercellen en fibroblasten, afkomstig kunnen zijn van mesenchymale stam cellen (MSCs) uit het beenmerg. Er is aangetoond dat MSCs kunnen uitgroeien tot cellen die specifieke kenmerken van pericyten en fibroblasten hebben. Aangezien pericyten en fibroblasten een belangrijke rol spelen in het bevorderen van tumorgroei, wijzen deze gegevens dus op een mogelijke cruciale rol van MSCs in de ontwikkeling van kanker.

Aan het begin van dit onderzoeksproject, zijn er in muizenstudies inderdaad enkele aanwijzingen gevonden dat MSCs tumorgroei kunnen bevorderen. Echter, het mechanisme waardoor MSCs tumorgroei kunnen stimuleren, was nog niet bekend.

Doel van het onderzoek

Het doel van het onderzoek beschreven in dit proefschrift was om vast te stellen of beenmergafkomstige mesenchymale stam cellen (MSCs) de groei van lymphoma bevorderen, om te onderzoeken wat het mechanisme hierachter is en of we de effecten van MSCs op tumorgroei kunnen remmen.

Resultaten en discussie

In *hoofdstuk 2* worden verschillende typen uit het beenmerg afkomstige cellen besproken, waarvan is aangetoond dat ze tumorgroei kunnen bevorderen. Tevens wordt de potentiële rol van MSCs in het manipuleren van de micro-omgeving van de tumor besproken, evenals de mogelijkheid om MSCs te gebruiken als transportmiddel voor therapeutische middelen tegen kanker.

In *hoofdstuk 3* is beschreven dat we het medicijn PTK787/ZK 222584 (Vatalanib, PTK) hebben getest om te onderzoeken of we de groei en functie van MSCs kunnen remmen. Deze studie wijst uit dat, als we PTK toedienen aan celkweken van MSCs, de groei van MSCs wordt geremd en er meer MSCs dood gaan. PTK is oorspronkelijk ontworpen om de vorming van bloedvaten (endotheel cellen) te remmen. In klinische trials is behandeling van kankerpatiënten met PTK al effectief gebleken in het remmen van tumorgroei door het remmen van de vorming van bloedvaten in de tumor. Gecombineerde strategieën, waarbij meerdere componenten in de micro-omgeving van de tumor geremd worden, zijn van groot belang voor het verbeteren van de bestaande therapieën tegen kanker. Onze resultaten laten zien dat PTK ook MSCs kan remmen en dat PTK dus één van de medicijnen zou kunnen zijn die gebruikt kan worden om de potentiële bijdrage van MSCs aan tumorgroei te remmen.

In *hoofdstuk 4* hebben we beschreven dat, in ons muismodel, de aanwezigheid van MSCs in de micro-omgeving van de tumor inderdaad de tumorgroei bevordert. In de literatuur worden tegenstrijdige resultaten beschreven met betrekking tot MSCs en tumorgroei: MSCs zouden tumorgroei bevorderen maar ook inhiberen. Een verklaring zou kunnen zijn dat het effect van MSCs op tumorgroei afhankelijk is van het type tumor. Anderzijds zou de verklaring kunnen liggen in de ratio MSCs vs. tumorcellen die gebruikt is. Zo is er in een muizenstudie, waarbij verschillende verhoudingen van Renca tumorcellen en MSCs zijn getest, gevonden dat bij een hoge verhouding van MSCs en tumorcellen (10:1) de tumorgroei bevorderd werd, terwijl bij een lage verhouding van MSCs en tumorcellen (1:10) de tumorgroei juist geremd werd. In ons onderzoek zagen we dat een lage verhouding van MSCs en Daudi tumorcellen (1:10) de tumorgroei bevorderde, wat duidt op een relatief hoge gevoeligheid van de Daudi tumor cellijn voor de groeibevorderende effecten van MSCs.

In onze studie hebben we gekeken naar mogelijke directe effecten van MSCs op tumorcellen. Hiervoor hebben we een kweeksysteem gebruikt waarin we tumorcellen samen met MSCs hebben laten groeien. Uit deze proeven is gebleken dat de groei van de tumorcellen deels bevorderd wordt door groeifactoren, die geproduceerd worden door MSCs, maar vooral door direct cel-cel contact tussen tumorcellen en MSCs. Tevens hebben we laten zien dat MSCs de tumorcellen kunnen beschermen tegen celdood. Bij direct cel-cel contact spelen integrines en fibronectines een grote rol. Het is bekend dat MSCs integrines tot expressie brengen en veel extracellulaire matrix moleculen (ECM) kunnen produceren. Studies hebben aangetoond dat binding van tumorcellen aan integrines of ECM de overleving van deze tumorcellen bevordert. Interessant genoeg, wordt, in ons onderzoek, het beschermende effect van MSCs op de tumorcellen teniet gedaan wanneer we PTK toedienen aan de celkweken van MSCs. Ook wanneer we muizen behandelen met PTK, lijkt het erop dat de effecten van MSCs op tumorgroei teniet kunnen worden gedaan.

Onze resultaten suggereren verder dat MSCs de vorming van bloedvaten in de tumor stimuleren. Er is in de literatuur beschreven dat MSCs kunnen uitrijpen tot endotheel-achtige en pericyt-achtige cellen en dat ze in die vorm kunnen bijdragen aan de

vorming van bloedvaten. Daarnaast is recentelijk aangetoond dat MSCs ook als fibroblast-achtige cellen kunnen bijdragen aan tumorgroei. Hoewel MSCs, in onze studie, wel in de tumor aangetoond konden worden, waren het te weinig om vast te kunnen stellen tot welk type cel ze uitgerijpt waren en in welke hoedanigheid ze hun bijdrage hebben geleverd aan tumorgroei.

In *hoofdstuk 5* hebben we onderzocht of vascular endothelial growth factor-A (VEGFA), een groeifactor die in veel tumoren geproduceerd wordt, de migratie van MSCs naar de tumor bevordert. Hiervoor hebben we een Daudi tumor cellijn gemaakt, die ongeveer 90 keer zoveel VEGFA produceert als de originele tumor cellijn. In deze studie hebben we, 24 uur na het inspuiten van de MSCs in de bloedbaan van de muizen, geen MSCs kunnen terugvinden in de tumoren, ook niet in de tumoren die VEGFA tot overexpressie brachten. Wel zien we heel veel MSCs in de longen en enkele MSCs in de milt. Dit resultaat suggereert dat, in ons model, MSCs niet specifiek naar tumoren migreren en dat VEGFA waarschijnlijk geen belangrijk signaalmolecuul is voor de migratie van MSCs naar tumoren.

Nu blijft er een belangrijke vraag over, want: hoe aannemelijk is het nu dat MSCs spontaan vanuit het beenmerg naar tumoren migreren? Studies tonen aan dat MSCs aanwezig kunnen zijn in de bloedbaan van verschillende zoogdiersoorten, waaronder de mens. Echter de aantallen MSCs zijn bijzonder laag en sommige studies hebben helemaal geen MSCs kunnen aantonen in menselijk bloed. Er zijn aanwijzingen in muizen en ratten dat MSCs gemobiliseerd kunnen worden uit het beenmerg en naar het hart kunnen migreren in reactie op een hartinfarct. Ook in een model voor osteogenesis imperfecta (een afwijking van de botten) is spontante migratie van MSCs van het ene bot naar het andere bot aangetoond. Deze gegevens suggereren dat MSCs inderdaad onder bepaalde omstandigheden vanuit het beenmerg naar de bloedbaan kunnen migreren. Echter, direct bewijs van spontane mobilisatie van MSCs uit het beenmerg en migratie naar tumoren ontbreekt tot op heden. Om dit te onderzoeken, zou het interessant zijn om een muismodel te ontwerpen waarbij enkel de MSCs in het beenmerg gemarkeerd zouden zijn. Als na opwekking van tumorgroei in deze muizen gemarkeerde MSCs in de micro-omgeving van de tumor gevonden worden, dan moeten ze afkomstig zijn uit het beenmerg, wat zou bewijzen

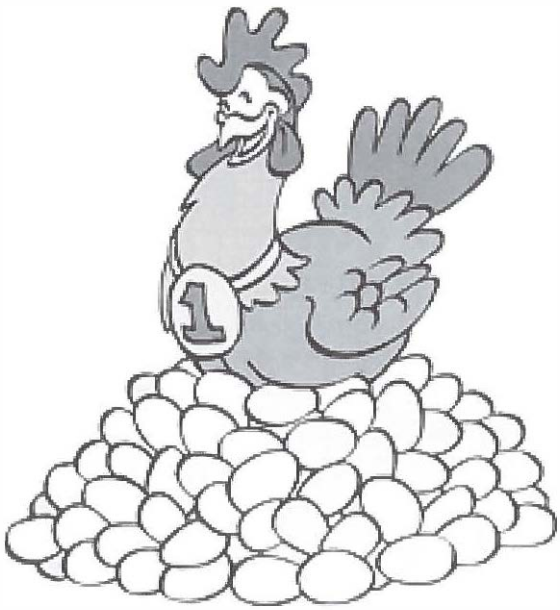
dat MSCs spontaan vanuit het beenmerg naar tumoren kunnen migreren. Verder zou het interessant zijn om, in de mens, te onderzoeken of MSCs die uit het beenmerg afkomstig zijn ook daadwerkelijk bijdragen aan tumorgroei. Echter, aangezien MSCs geen specifieke marker hebben waarmee ze van andere cellen te onderscheiden zijn, zal het erg lastig zijn om MSCs aan te tonen in de mens.

In *hoofdstuk 6* hebben we beschreven dat VEGFA de groei van de tumor bevordert en dat dit gepaard gaat met een significante toename aan bloedvaten. Deze studie wijst uit dat groeifactoren, die geproduceerd worden in de micro-omgeving van de tumor, een prominente rol spelen in de toegenomen tumorgroei als gevolg van VEGFA. Onze resultaten suggereren hierbij een rol voor STAT eiwitten en $p27^{KIP1}$ in de tumorcellen. Interessant genoeg vonden we, zowel in de tumoren met MSCs (hoofdstuk 4) als in de tumoren met VEGFA, dat de hoeveelheid actief $p27^{KIP1}$ in de tumorcellen afgenomen was vergeleken met controle tumoren. $p27^{KIP1}$ is een van de cruciale moleculen die de cel cyclus reguleren; een afname van $p27^{KIP1}$ gaat gepaard met een toename van celdeling. Dit betekent dat de afname van $p27^{KIP1}$ in de tumoren de toegenomen groei van tumoren met MSCs en tumoren met VEGFA zou kunnen verklaren. Dit resultaat suggereert dus een belangrijke rol voor $p27^{KIP1}$ in de tumor cellijn die wij gebruikt hebben voor onze studies.

Conclusie

Het onderzoek gepresenteerd in dit proefschrift benadrukt de rol van de micro-omgeving van de tumor (inclusief MSCs) in de groei en ontwikkeling van tumoren en onderschrijft het de relevantie van het remmen van de effecten van de micro-omgeving van de tumor op tumorgroei als aanvullende strategie in de behandeling van kanker. Onze resultaten geven meer inzicht in de manieren waarop MSCs tumorgroei bevorderen. Meer onderzoek is nodig om de specifieke moleculen te identificeren die betrokken zijn bij de interacties tussen MSCs en tumorcellen. Dit zal informatie geven over welke specifieke interacties van belang zijn om te blokkeren in de behandeling van kanker. Tevens, onthullen de resultaten van ons onderzoek een belangrijke rol voor $p27^{KIP1}$ in het bevorderen van de groei van de Daudi Burkitt

lymphoma cellijn in reactie op omgevingsfactoren, zoals de aanwezigheid van MSCs of VEGFA. Dit duidt erop dat $p27^{KIP1}$ en eiwitten die de activiteit van $p27^{KIP1}$ reguleren belangrijke doelwit-moleculen (targets) kunnen zijn in de behandeling van Burkitt lymphoma. Toekomstig onderzoek zal hier uitsluitsel over moeten geven. Tenslotte zal toekomstig onderzoek moeten uitwijzen of MSCs inderdaad vanuit het beenmerg naar tumoren kunnen homen, en hoe relevant het zal zijn om de bijdrage van uit beenmerg afkomstige MSCs aan tumorgroei te remmen.



Het ei is gelegd!!!

Woord van dank

Beste allemaal

Nu, aan het einde van deze reis door onderzoeksland, wil ik iedereen die hieraan heeft bijgedragen heel erg hartelijk bedanken. Ten eerste wil ik mijn promotor Willem Kamps en co-promotor Eveline de Bont bedanken voor de kans die jullie me hebben gegeven om aan dit avontuur te beginnen. Eveline, dank voor je kritische blik en je besmettelijke enthousiasme. Ik zal nooit vergeten hoe belangrijk een goede focus is ("focussen Berber: één ding tegelijk") voor het voltooien van een project van deze omvang. Graag wil ik mijn collega's van de Kinderoncologie bedanken, die mij hebben gesteund en geholpen tijdens dit traject: Jessica, Arja, Frank, Tiny, Jenny, Kim, Alida, Henk-Marijn, Erik, Neeltje, Mariska, Bart en Hendrik. Bedankt voor de praktische ondersteuning, voor de gezelligheid en voor het klankbord dat jullie soms voor me waren. Natuurlijk wil ik ook de collega's van de Hematologie bedanken voor de gezelligheid en ondersteuning. De labdagen, het uitstapje naar de klimwand, maar vooral ons grote Wampex-avontuur zal ik nooit vergeten! Bedankt allemaal! Verder wil ik alle mensen van het Centraal Dieren Laboratorium bedanken voor hun bijdrage aan mijn muizenproeven en ook wil ik Geert Mesander bedanken voor zijn hulp bij het FACSen.

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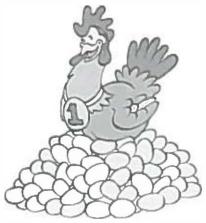

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Groeten, Berber.

Curriculum Vitae

Berber Dorienke Roorda was born on December 13th, 1979 in Emmeloord. She completed pre-university education (Gymnasium, Hondsrugcollege, Emmen) in 1997. Subsequently, she enrolled to study Biology at the University of Groningen and obtained her Bachelor degree Cum Laude in 1998. Her first intern undergraduate research project was performed at the department of Animal Physiology under supervision of Prof. Dr. Anton Scheurink. This project focussed on potential physiological changes in response to long-term physical workloads in rats. Her second research project was at the Novo Nordisk Company in Copenhagen, Denmark, where she investigated the usability of rats prone to diet-induced obesity as a model to study type 2 diabetes mellitus under supervision of Dr. Stephan Bouman and Dr. Christian Lehn Brand. At the end of 2001, she obtained her Master degree Cum Laude. In March 2002, she attended a research project at the department of Human Biology at the University of Maastricht on the role of UCP3 in glucose metabolism under supervision of Dr. Patrick Schrauwen and Dr. Matthijs Hesselink. In September 2005, she moved back to Groningen to start her PhD-project (this thesis) at the department of Pediatric Oncology of the Universitary Medical Center Groningen under supervision of Prof. Dr. Willem Kamps and Dr. Eveline de Bont. Since September 2009, she is employed as teacher at the department of Physiotherapy of the School of Health Sciences of the Hanze University of Applied Sciences, Groningen.

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